Isolation and characterisation of *three rows*, a gene essential for mitotic chromosome disjunction in *Drosophila melanogaster*

A thesis submitted for the degree of Doctor of Philosophy

by

Ulrik Peter John, B.Sc.(Hons), M.Sc.

Departments of Biochemistry and Genetics
University of Adelaide
Adelaide, S.A., 5005
Australia

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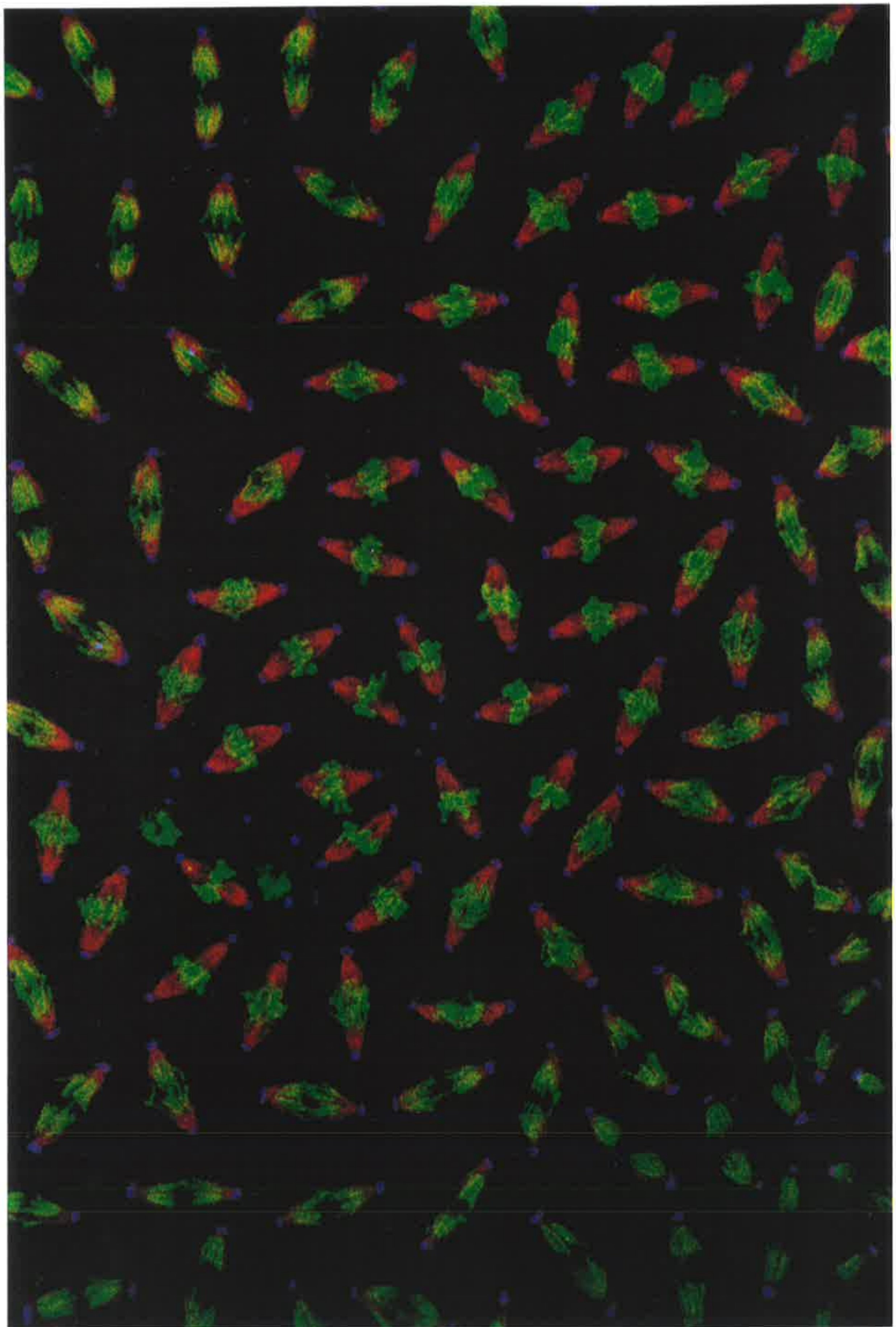
Confocal image of syncytial blastoderm embryo showing nuclei in metaphase (diagonal from top right to bottom left) proceeding into anaphase. Chromosomes shown in green, microtubules (mitotic spindle) in red, and centrosomes in blue. Some nuclei have become dissociated from pairs of centrosomes.



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Abstract

Zygotic expression of the *three rows* (*thr*) gene of *Drosophila melanogaster* is required for normal cell proliferation during embryogenesis (D'Andrea et al., 1993). Mitotic defects in *thr* mutant embryos begin during mitosis 15, and all subsequent divisions are disrupted. Chromosome disjunction and consequently cytokinesis fail during these defective mitoses, although the initial mitotic processes, and subsequent cell cycle progression are not affected.

The *thr* gene has been identified, in a chromosome walk from the nearby *grainyhead* gene, by correlation with a P element insertional polymorphism in the hybrid dysgenic allele thr^{BH}. Cloning of *thr* was confirmed by complementation of lethality in a homozygous mutant background, with a genomic fragment from the region. The P element insertion site has been defined by nucleotide sequencing and shown to interrupt a long ORF corresponding with cDNA clones isolated from early embryonic libraries. *thr* encodes a 1,379 aa protein that shares no extended sequence similarity with known proteins.

thr mRNA is present as abundant, maternally conferred transcript which degrades at the time of cellularisation. At this and all subsequent times during development, zygotic expression correlates with mitotic proliferation. These observations suggest that the embryonic phenotype results from exhaustion of the maternal *thr* contribution and does not reflect a developmentally restricted requirement for *thr* function. The delay in the manifestation of the mutant phenotype until cycle 15 is believed to reflect persistance of protein derived from maternal mRNA.

Immunostaining of embryos with three rows specific antibodies has revealed a cell cycle dependent pattern of localisation, consistent with the defect in chromosome disjunction observed in mutants. Three rows, undetectable in metaphase, is localised to the chromosomes in anaphase, initially to the region of the presumptive kinetochore.

By the criteria of low stringency hybridisation to genomic Southern and library filters, sequences homologous to *thr* can only be detected in *Drosophila* species thought to have shared a common ancestor with *D. melanogaster* up to 20 mya. A homologue of *thr* isolated from *D. erecta* encodes a protein with 88.3% sequence identity with three rows of *D. melanogaster* over the common region.

Statement

This work contains no material which has been accepted for the award of any other degree of diploma in any university of other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent for this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Ulrik John, 10/1/95

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Chapter 1 (G2): Introduction

This study concerns the characterisation of a gene, *three rows*, whose mutant phenotype of failure of chromosome disjunction in anaphase, is indicative of an essential but unknown function in mitosis.

1.1 General principles of mitosis

Mitosis is the process by which eukaryotic cells faithfully segregate their duplicated genomes into two complete sets, usually at cell division. Mitosis occurs during the stage of the eukaryotic cell cycle (Figure 1.1), referred to as M phase, and follows replication of the chromosomal content in S phase. Interspersed between these two phases are the "Gap" phases, G1 and G2 (Figure 1.1), during which commitment to, and preparation for, the ensuing S and M phases occurs.

Mitosis can be viewed as the result of interactions between three major multicomponent systems: i) the spindle, a microtubule (MT) based machine whose bipolar organisation achieves the equipartition of ii), highly compacted chromosomes with specialised structures upon them for engaging the spindle, and iii), a self governing molecular oscillator which regulates the first two systems by controlling the level and activity of their constituent proteins. The molecular oscillator will be described in section 1.2.

Despite obvious differences in the appearance of spindles and chromosomes in various eukaryotes it is believed that the fundamental mechanisms of mitosis have been conserved in evolution from yeast to humans. Our present understanding of mitosis has come from the integration of data from genetically tractable organisms, such as the fission and budding yeasts and *Drosophila melanogaster*, with cytological observations predominantly from vertebrate cells.

Cytogeneticists studying mitosis have defined a series of sequential stages: prophase, prometaphase, metaphase, anaphase and telophase (Figure 1.2). In *prophase*

Figure 1.1 The eukaryotic cell cycle (from Murray and Hunt, 1993).

See text for details.

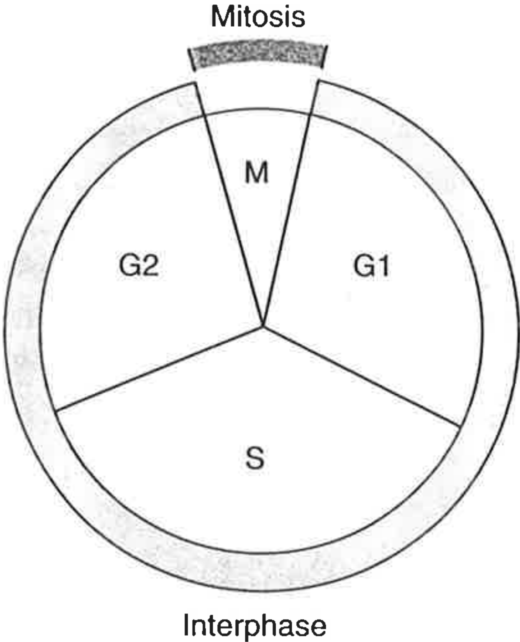
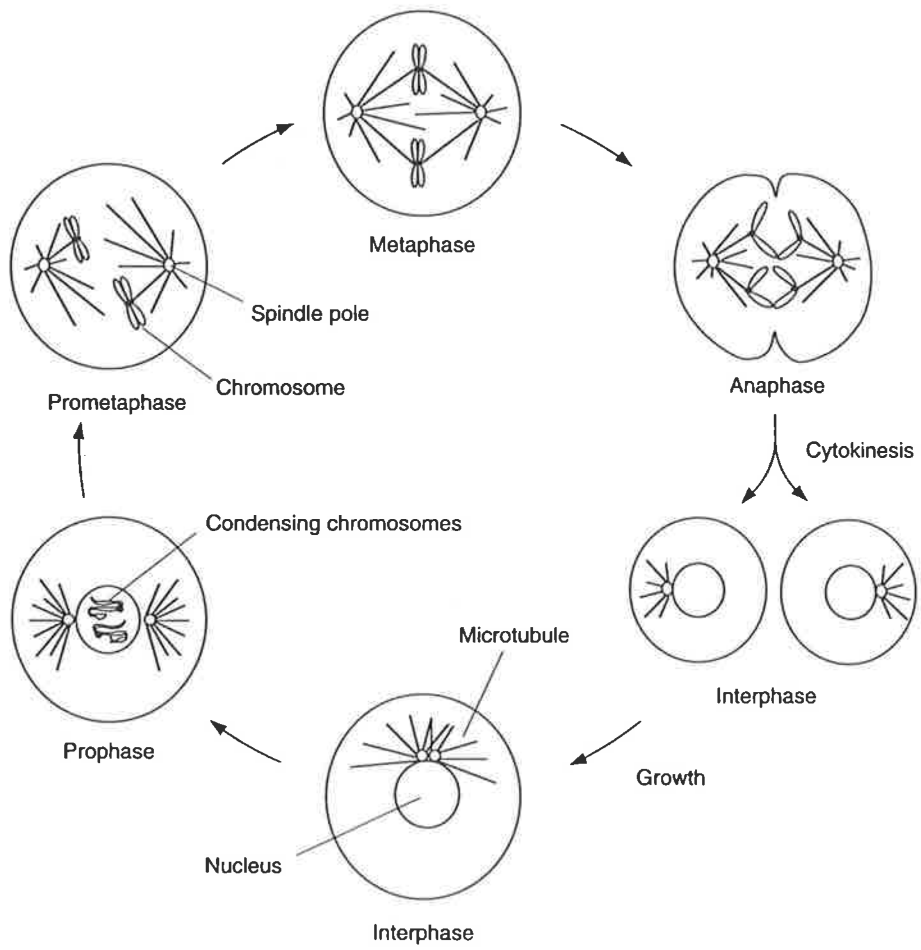

Mitosis
M
G2
G1
S
Interphase

Figure 1.2 Stages of mitosis (from Murray and Hunt, 1993).

See text for details.


Metaphase
Spindle pole
Chromosome
Prometaphase
Anaphase
Cytokinesis
Condensing chromosomes
Microtubule
Interphase
Prophase
Growth
Nucleus
Interphase

chromosomes that have been replicated undergo condensation. The chromosomes are captured by the mitotic spindle in *prometaphase* which positions them at the spindle equator at *metaphase*. In *anaphase* the sister chromatids separate and are transported by the spindle to opposite ends of the cell. Finally in *telophase* the chromosomes decondense and reestablish their *interphase* (a collective term for G1, S and G2 phases) state.

1.1.1 Microtubule organising centres

The microtubule organising centre (MTOC) is the major nucleator of MTs in both mitosis and interphase. Despite the structural dissimilarity between the MTOCs of fungi (the spindle pole body) and animals (the centrosome) they do have protein components in common. However it is yet to be demonstrated that they are homologous structures by virtue of common ancestry.

The MTOC of yeast is the disc shaped Spindle Pole Body (SPB). Consistent with the closed form of mitosis in yeast the SPB remains embedded in the nuclear envelope throughout the yeast cell cycle where it simultaneously nucleates spindle MTs from its intranuclear surface, and cytoplasmic MTs from the converse side. The SPB is a trilaminar structure with its central layer contiguous with the nuclear membrane and MTs emanating from the amorphous surface layers.

The centrosome of animal cells is a poorly defined cytoplasmic organelle consisting of two orthogonally arranged centrioles surrounded by the amorphous pericentriolar material (PCM) from which MTs emanate. Centrioles are related to, and in some circumstances interchangable with, the basal body of flagellae. Each centriole is composed of a short barrel of MTs in a "9+0" arrangement. It is believed that centrioles are unable to arise *de novo*, their duplication being template driven by a preexisting centriole. While various centrosomal components have been identified by immunological means (Kellogg et al., 1989; Balczon and West, 1991) our knowledge of their arrangement and function is scant.

Evidence of shared function in the MTOCs of fungi and animals has come from the identification of an evolutionarily conserved minor tubulin, γ-tubulin. γ-tubulin was originally identified in *Aspergillus nidulans* as a suppressor of a β-tubulin mutation

(Oakley and Oakley, 1989). Localisation of γ-tubulin to the SPB and demonstration that mutations in γ-tubulin in *A. nidulans* are severely compromised in MT formation led to the hypothesis that γ-tubulin may act as the site of MT nucleation (Oakley et al., 1990). Furthermore, this function appears to be highly conserved, as γ-tubulin has been detected immunologically in the PCM in mammalian cells (Stearns et al., 1991), and cloned in *Schizosaccharomyces pombe*, *D. melanogaster*, *Xenopus laevis*, mouse and humans (Horio et al., 1991; Stearns et al., 1991; Zheng et al., 1991; Joshi et al., 1992). γ-tubulin has also been shown to be part of a complex with other centrosomal proteins in *D. melanogaster* (Raff et al., 1993). Although γ-tubulin's direct interaction with the minus ends of MTs is as yet unproven, the demonstration that antibodies directed against it inhibit the nucleation of new MTs but do not affect extant ones (Joshi et al., 1992), is highly suggestive.

1.1.2 The assembly of the spindle

The assembly of a *bipolar* spindle is tied irrevocably to duplication and separation of the MTOCs. Studies in fungi and mammals have revealed similar proteins involved in both of these processes.

The replicate SPB is formed adjacent to the preexisting one, first appearing as a "satellite" on the cytoplasmic side of the nuclear envelope early in G1. Two daughter SPBs arise following passage through "Start" (Hartwell et al., 1974), the transition point late in G1 in which the cell becomes committed to undergo a complete cell cycle. The daughter SPBs remain joined by a bridge until separated in S phase. The assembly of SPBs is poorly understood, in part because few mutants have been found that affect the process (Kilmartin, 1994). The product of one gene required for SPB duplication in *Saccharomyces cerevisiae*, *MPS1*, is an essential protein kinase (M. Winey, pers. comm.). Another gene, *CDC31,* encodes a low molecular weight calcium-binding protein (Baum et al., 1986) of the EF-hand superfamily. The ubiquitous homologue of *CDC31*, centrin, has been isolated in protozoans (Huang et al., 1988), higher plants (Zhu et al., 1992) and humans (Errabalou et al., 1994), and localised to the centrosome (Huang et al., 1988; Errabalou et al., 1994).

In most animal cells centrosome duplication begins in G1 when the centrioles cease their orthogonal arrangement and move slightly apart. Centriole replication is initiated early in S phase, with the appearance of the procentriole perpendicular to the base of each existing centriole, and is completed in G2 phase.

The MTs nucleated by MTOCs are polar structures with plus and minus ends defined by their polymerisation properties and the asymmetry of their tubulin subunits. The more stable minus end is proximal to the MTOC and the dynamic plus end is distal. MTs are highly unstable structures existing in two states, either shrinking or growing. Transitions from the shrinking to growing state are known as "rescue" and the converse as "catastrophe". Because of the dynamic instabity of MTs the mitotic spindle is capable of undergoing rapid changes in structure. Spindle assembly at the onset of mitosis is allied to an increase in the catastrophe frequency relative to that of rescue (Belmont et al., 1990), leading to the production of increased numbers of short, spindle MTs.

Many different types of proteins associate with MTs. Some MT associated proteins (MAPs) modify MT dynamics while others are mechanochemical motors. Motor proteins couple energy from nucleotide hydrolysis to movement along the MT. Different motor proteins may have different "adaptors" that enable them to engage and move subcellular components, or to interact with other MTs and thus exert tension (Goldstein, 1991). Reflecting the polarity of their substrate, motor proteins usually move unidirectionally being classified as either "plus end" or "minus end" directed.

Following their duplication MTOCs must be separated for a functional bipolar spindle to formed. MTOC separation is a MT dependent process mediated by members of a class of minus end directed molecular motors in both higher and lower eukaryotes. The "bimC" family are phylogenetically distinct (Goodson et al., 1994) members of an abundant, multifunctional class of Kinesin Related Proteins (KRPs) (Endow and Hatsumi, 1991). Mutants of bimC in *A. nidulans* (Enos and Morris, 1990), cut7 in *S. pombe* (Hagan and Yanagida, 1990), Cin8p and Kip1p in *S. cerevisiae* (Hoyt et al., 1992), KLP61F (encoded by the *urchin* gene) in *D. melanogaster* (Heck et al., 1993) have MTOC separation blocked. The same defect has been observed in *in vitro* spindle assembly assays with antibodies against the *X. laevis* KRP Eg5 (Sawin et al., 1992a).

Immunolocalisation of cut7, Cin8p, Kip1p and Eg5 (Hagan and Yanagida, 1992; Hoyt et al., 1992; Roof et al., 1992; Sawin et al., 1992a) to MTs between the MTOCs is consistent with a hypothesis that MTOC separation is conferred by sliding of antiparallel MTs, driven by KRPs at the spindle midzone. Another KRP, human MKLP, with antiparallel MT sliding activity *in vitro* is localised to the spindle midzone (Nislow et al., 1992) but is not a member of the bimC family (Goodson et al., 1994). Evidence of an additional mechanism for MTOC separation in anaphase in vertebrates is discussed in 1.1.5 below.

KRPs are also involved in maintaining the integrity of the spindle once it has been assembled, possibly by the generation of opposing forces. Thus, deletion of Cin8p and Kip1p which causes collapse of the spindle can be partially suppressed by loss of function in another KRP Kar3p (Saunders and Hoyt, 1992). This is consistent with recent data that Kar3p functions as a minus end directed motor (Endow et al., 1994). Similarly, in *A. nidulans* defective bimC can be compensated for by deletion of klpA, a Kar3p related KRP (O'Connell et al., 1993).

The contribution of motor proteins to force generation in chromosome movement is discussed in 1.1.5 below.

1.1.3 Centromere and kinetochore structure

Kinetochores are plate shaped complexes of specialised proteins that bind to specific centromeric DNA sequences, enabling chromosomes to engage the spindle. An apparent lack of conservation of kinetochore proteins and centromeric sequences throughout evolution (Bloom, 1993) have hindered the gaining of functional insights. To date the most productive source of characterised kinetochore proteins has been humans, whilst the only defined centromeric DNA sequence is that of *S. cerevisiae*.

The centromeric DNA of *S. cerevisiae* is only 125 nt in length and consists of three distinct sequence elements *CDEI, CDEII* and *CDEIII* (Fitzgerald-Hayes et al., 1982). All three elements are essential but the 25bp *CDEIII* is absolutely required for centromeric function (Ng and Carbon, 1987). *CDEIII* is specifically bound, in a phosphorylation dependent manner, by CBF3, a 240 kDa complex of three major proteins CBF3A, B and C, and some minor proteins (Lechner and Carbon, 1991). The complex

harbours MT minus end directed motor activity (Hyman et al., 1992) for which the minor component Kar3p is probably responsible (Endow et al., 1994; Middleton and Carbon, 1994). Genes encoding the CBF3A and C proteins have been cloned, both having been independently isolated in genetic screens for mitotic defects (see Bloom, 1993). Two of the other minor components are CBF5p a putative MAP, and DNA topoisomerase II (Jiang et al., 1993).

Investigations of human centromere and kinetochore structure have exploited autoantibodies from patients with the syndrome CREST (Calcinosis, Raynaud's phenomenon, Esophageal (sic) dismotility , Schlerodactly, and Telangiectasia) (Moroi et al., 1980). These autoantibodies react with four distinct kinetochore proteins (CENPs) (Earnshaw and Rothfield, 1985) and also two from the inner centromere (INCENPs) (Earnshaw and Cooke, 1991). Another protein, CENP-E (Yen et al., 1991) has subsequently been added to the group. The corresponding genes have been cloned and their products characterised (Table 1.1).

Table 1.1 Mammalian kinetochore proteins (from Bloom, 1993).

Protein	Motifs	Phenotype
CENP-A	Histone-like	
CENP-B	Acidic serine-rich region	G2-M arrest
CENP-C	Hydrophilic and highly basic	
CENP-D	GTP-binding (RCC1 homolog)	
CENP-E	Kinesin-like microtubule based motor	Metaphase arrest
INCENPA	Coiled-coil domain	
INCENPB	Coiled-coil domain	

The characterisation of centromeric sequences in mammals has been greatly hindered by their enormous size. For example the centromere of the smallest human

chromosome, 21, may occupy more than 5 Mb of its approximately 40 Mb total (Earnshaw and Tomkiel, 1992). Short functional centromeric sequences may reside amongst long stretches of repetitive DNA that characterise centromeric regions or else the repetitive sequences themselves may confer aspects of centromere function. Evidence for the latter hypothesis comes from the demonstration that the consensus binding site for CENP-B, the CENP-B box, is a 17 nt sequence found in α-satellite DNA (Muro et al., 1992). α-satellite DNA is present in abundance in mammalian centromeres, as higher order repeats of a 171 nt monomer. However CENP-B binding is clearly not sufficient for centromere function as it has been detected in inactive centromeres (Earnshaw et al., 1989).

The presence of other proteins at vertebrate kinetochores has been demonstated, most notably the minus end directed motor dynein. Anti-dynein antibodies stain the kinetochores of mitotic cells (Pfarr et al., 1990; Stuer et al., 1990) and isolated chromosomes (Wordeman et al., 1991). KRPs have also been detected immunologically (Sawin et al., 1992b) and the human KRP CENP-E localises to kinetochores during prometaphase and metaphase (Yen et al., 1992). These results are consistent with *in vitro* kinetochore motility assays which have identified two different motor activities of opposite polarities (Hyman and Mitchison, 1991b).

1.1.4 Chromosome motion in mitosis

Because of the inadequacies of cytology in yeast (closed mitoses, low levels of chromosome condensation, and minimalist spindles) descriptions of chromosome behaviour in mitosis have come almost exclusively from vertebrate cells. In particular newt lung epithelial cells have been highly favoured because of their large chromosomes, flatness and optical clarity. These studies have revealed that chromosomes undergo continuous oscillatory movements relative to the spindle poles throughout mitosis (Skibbens et al., 1993), and have lent weight to the concept of the "smart kinetochore" (Mitchison, 1989).

Chromosomes, when first attached to the spindle, are mono-orientated. Kinetochores initially interact with the lateral surface of one of the MTs extending from, or retracting to, the spindle poles. Immediately upon attachment the chromosome moves

rapidly (an order of magnitude faster than all subsequent movements (Rieder and Salmon, 1994)) along the side of the MT towards the spindle pole from which it is nucleated (Rieder and Alexander, 1990). As it nears the pole it slows and the kinetochore encounters more MTs, the *ends* of which it now interacts with. These MTs are known as kinetochore MTs (kMTs). Oscillatory movements now commence which are characterised by abrupt transitions in direction termed "directional instability" (Skibbens et al., 1993). From the distortion of the centromeric region it has been inferred that force is generated by alternate pushing or pulling movements at the kinetochore (Skibbens et al., 1993).

In a process that may be aided by the oscillations, the other unoccupied kinetochore eventually captures a MT (usually) from the opposite pole. The bi-orientated chromosome now undergoes "congression" to take up a position on the spindle equator at metaphase. Bi-orientated chromosomes also continue oscillatory movements in a manner which suggests "cooperative switching" (Skibbens et al., 1993). If one kinetochore is moving polewards, then 75% of the time its sister is moving away from its pole. The mechanism by which the behaviour of one kinetochore can influence that of the attached one is unknown, but it is argued the "smart" kinetochores can sense tension at the sites of kMT attachment and alter the frequency of phase transitions accordingly (Skibbens et al., 1993).

In anaphase the sister chromatids disjoin and are moved to opposite ends of the cell. Anaphase consists of two types of movement: anaphase A and anaphase B. In anaphase A the sister chromatids move towards the spindle poles concommitant with kMT shortening, whilst in anaphase B the spindle elongates and the poles move apart. In animal cells anaphase A and B occur simultaneously. In *S. pombe* both spindle elongation and kMT shortening have been observed concommitant with sister chromatid separation (Ding et al., 1993).

1.1.5 Force generation in mitosis

The forces that move chromosomes in mitosis remain an area of contention. It has been demonstated that under certain conditions either mechanochemical motor activity, or

MT dynamics at either the plus or minus ends can produce sufficient force to realistically account for observations of chromosome movements during mitosis.

As discussed in section 1.1.3, immunological data and results from *in vitro* experiments localise both plus and minus end directed motor activity to the kinetochore. Certainly, the initial movement of a mono-orientated chromosome poleward along the lateral surface of a MT is difficult to reconcile, other than with the action of a minus end directed kinetochore motor. On the basis of its localisation to the kinetochore, and its kinetic properties, cytoplasmic dynein has been proposed as the motor responsible for this movement (Rieder and Alexander, 1990). However in yeast, disruptions of the dynein gene merely perturb spindle positioning (Li et al., 1993), and anti-dynein antibodies have no effect on chromosome movement in mammalian cells (Vaisberg et al., 1993).

MT dynamics alone can also be sufficient to generate bidirectional chromosome movement. MT polymerisation at the kinetochore can induce chromosome movement away from the poles (Shelden and Wadsworth, 1992), and in the absence of ATP kinetochores can remain bound to a MT as it depolymerises (Koshland et al., 1988). Furthermore, fluorescent marking of spindle MTs have revealed there is a continuous flux of MTs polewards in metaphase and early anaphase caused by MT disassembly at the pole (Mitchison and Salmon, 1992). Such flux could account for up to 37% of chromosome to pole movement (Mitchison and Salmon, 1992).

The resolution of the relative contributions of kinetochore motors and MT dynamics may come from the observation that kinetochores interact almost exclusively with the ends of MTs. In such an arrangement kinetochore movement must be coordinated with MT plus end polymerisation or depolymerisation. Thus MT motors may simply function to maintain contact with the labile MT ends. MT polymerisation kinetics would then determine the velocity of chromosome movement. Alternatively, the motor proteins themselves could regulate MT dynamics.

Another less well characterised MT mediated force producing mechanism is the astral ejection force or "polar wind". The spindle exerts a pressure, demonstrated by the active transportation away from the pole of chromosome arms severed with a laser microbeam (Rieder et al., 1986). Astral ejection forces may contribute to the preanaphase,

away from pole, movement of oscillating chromosomes (Cassimeris et al., 1994). The nature of the force is unknown but it could simply result from the impact of growing MT ends (Rieder et al., 1986).

Polewards movement of sister chromatids during anaphase A is believed to be conferred by the same forces as in earlier stages, as chromosomal oscillations can still be observed (Rieder and Salmon, 1994). Clearly however there is a shift in bias to pole directed movement. This may simply be a product of abolishing the connection between chromatids and as the magnitude of the astral ejection force is proportional to the chromosomal area, the chromatid arms undergo sustained polewards movement (Rieder and Salmon, 1994). The changes in MT dynamics that accompany disassembly of the spindle in anaphase would hasten this process. Alternatively there may be regulatory changes at the kinetochore which alter MT dynamics or motor activity.(Hyman and Mitchison, 1991a; Rieder and Salmon, 1994).

Anaphase B spindle elongation is an analogous process to MTOC separation required for spindle assembly (Section 1.1.2). There are two hypotheses for how this may occur. Either the force is produced by antiparallel pushing of MTs in the spindle midzone, as shown in yeast SPB separation, or from pulling forces between the astral MTs and some peripheral anchor.

In lower eukaryotes there is evidence that the same forces are responsible for SPB separation in prophase and in anaphase. Anti KRP antibodies localise to the midzone of isolated diatom metaphase spindles and inhibit elongation (Hogan et al., 1992). Evidence also comes from a cold sensitive mutant of *S. cerevisiae*, that only forms spindle but not astral MTs, in which anaphase B is normal (Sullivan and Huffaker, 1992).

In vertebrates the latter mechanism clearly operates as centrosome migration continues when half spindle MT arrays no longer overlap (Waters et al., 1993). However both mechanisms probably contribute since spindle elongation occurs at a faster rate when there is overlap, implying the antiparallel MTs act as a ratchet to govern the rate and direction of centrosome separation (Waters et al., 1993). Minus end directed motors tethered in the cytoplasm could provide the force for the proposed astral pulling, and

antibodies against dynein have been shown to inhibit centrosome separation (Vaisberg et al., 1993).

One striking feature of mitotic force production is the many instances of seeming redundancy of the mechanisms employed. A number of examples of this have been discussed here. Thus: both MT dynamics or motor proteins may be adequate to move chromosomes, more than one motor protein may contribute to the same process as for Cin8p and Kip1p in *S. cerevisiae*, anaphase B is driven by both pushing and pulling mechanisms in vertebrates, and anaphase A and anaphase B both contribute to chromosome segregation. While such redundancy may simply reflect inadequacies in our understanding of these processes, it may also exist to enhance the accuracy of mitosis, a process with strong selection for high fidelity (Goldstein, 1993; Thomas, 1993)

1.1.6 Sister chromatid disjunction

At anaphase the linkages which have held sister chromatids together throughout mitosis are abolished almost simultaneously as a result of an unknown signal or reaction. These linkages are not only proximal to the centromere but extend the entire length of the chromosome (Rattner et al., 1988). Sister chromatid separation is a MT independent process in some systems (reviewed in Rieder and Palazzo, 1992) but this is evidently not the case in *D. melanogaster* (Gonzalez et al., 1991; Williams et al., 1992). However the dependence in *D. melanogaster*, of chromatid disjunction on spindle integrity, probably does not reflect a requirement for force production in disjunction but is a function of checkpoint surveillance (Section 1.1.7).

On the basis of their localisation to the region of contact between sister chromatids in metaphase, the human INCENPs have been proposed to function in chromatid separation (Cooke et al., 1987). However there is no direct evidence for this. Indeed INCENPS become redistributed to the spindle midzone significantly in advance of anaphase (Earnshaw and Cooke, 1991). By contrast the presence of another class of antigens, the CLiPs (Chromatid Linking Proteins) is correlated with chromatid association (Rattner et al., 1988).

One product whose activity is essential for sister chromatid separation is type II DNA topoisomerase (topo II). Eukaryotic chromosomes typically complete DNA replication with multiple intertwinings between the DNA strands of the two chromatids. Disjunction in such a state would be extremely deleterious. Topo II can resolve chromosome tangling by producing a double strand cut in the DNA of one chromatid, passing the DNA of the other through the cut, then religating. Disruption of topo II activity in yeasts, *X. laevis* and *D. melanogaster* leads to failure of chromatid separation and also causes defects in chromosome condensation (DiNardo et al., 1984; Uemura et al., 1987; Shamu and Murray, 1992; Buchenau et al., 1993).

1.1.7 Trouble shooting

To achieve the exceptionally high rates of fidelity ($<10^{-5}$ mistakes per cell division (Hartwell and Smith, 1985)) observed for chromosome segregation, eukaryotes have developed dependency relationships that couple anaphase to the successful completion of earlier events. Mechanisms exist to delay or block mitotic progression if a functional bipolar spindle has not been formed, if chromosomes are malorientated, or if kinetochores are defective.

Some, but clearly not all, of these arrests or delays are the product of "checkpoints" (Hartwell and Weinert, 1989), where an extrinsic surveillance mechanism monitors the successful completion of an earlier process. Checkpoints can be thought of as signal transduction pathways that *feed forward* to downstream events empowering them to proceed (Hartwell, 1991). Other arrests are simply a consequence of dependencies analogous to a substrate-product mechanism (Hartwell and Weinert, 1989), in which downstream events will not proceed in the absence of a pre-existing product or condition.

Prometaphase is prolonged to variable degrees (Kung et al., 1990) in cells that have been treated with agents that either disrupt or stabilise MTs, even at levels that produce no observable effects (Rieder and Palazzo, 1992; Jordan et al., 1993; Wendel et al., 1993). Prolongation is also observed in the absence of spindle bipolarity (Hunt et al., 1992).

Similarly, uncongressed chromosomes produced either by irradiation (Zirkle, 1970), micromanipulation (Rieder and Alexander, 1989), treatment with kinase inhibitors (Nicklas et al., 1993), or occurring naturally (Nicklas and Arana, 1992) delay anaphase onset.

Lesions in kinetochores also cause mitotic arrest. In *S. cerevisiae* a mutation in the centromeric DNA of one chromosome, in the presence of 32 normal centromeres, can retard mitosis (Spencer and Hieter, 1992), as does a mutation in the *CTF13* gene encoding one of the CBF3 kinetochore complex proteins (Doheny et al., 1993). Anaphase is also delayed in cells injected with a mixture of antibodies, against several CENP kinetochore proteins (Bernat et al., 1990), and against the kinetochore KRP CENP-E (Yen et al., 1991).

While many of these observed delays have been ascribed to the action of checkpoints, very few have satisfied the criteria of being mediated by an extrinsic mechanism that can be mutated or disrupted in order to bypass the delay. Assembly of a functional spindle is one process that is clearly subject to checkpoint monitoring. Mutants have been isolated in *S. cerevisiae,* the *bub*$^-$ and *mad*$^-$ strains, that fail to block or delay mitotic progression in response to inhibitors of MT polymerisation (Hoyt et al., 1991; Li and Murray, 1991).

It has been suggested that checkpoint monitoring of spindle assembly, and the arrests due to chromosome malorientation and kinetochore defects may be part of the same surveillance mechanism that specifically monitors the interaction of kinetochores with MTs (Zirkle, 1970; Murray and Hunt, 1993). Evidence for a possible mechanism of signalling kinetochore attachment to the spindle has come from the detection of a phosphorylated epitope expressed on kinetochores in prometaphase but lost at metaphase (Gorbsky and Ricketts, 1993). Most striking is the observation that a misaligned chromosome, capable of delaying anaphase onset, strongly expresses the phosphoepitope while the remaining chromosomes at the metaphase plate do not.

Interestingly no checkpoint mechanism for monitoring anaphase chromosome segregation have yet been discerned. Evidence that this process is not subject to monitoring comes from the occurrence of mutants which fail in disjunction yet undergo

subsequent DNA replication to become polyploid (Sections 1.3 and 1.5.1). Some dependency relationships however clearly operate on later events, as cytokinesis is often defective in the absence of disjunction. For instance, cytokinesis is still blocked in *bub* mutants in presence of MT inhibitors (Hoyt et al., 1991).

While the spindle assembly checkpoint is involved in troubleshooting at the terminal stages of mitosis other checkpoints control entry into mitosis. Checkpoint pathways have been genetically characterised in yeasts, that delay the onset of mitosis in the event of DNA damage or failure to complete replication (reviewed in Murray, 1992). Mutants have been isolated in *S. cerevisiae* and *S. pombe* that prevent arrest in response to damaged DNA, or to unreplicated DNA, and some that are defective in both pathways.

Checkpoint mechanisms are not a universal phenomena. Their absence is a feature of early embryogenesis in some organisms, including sea urchins (Sluder et al., 1994) and *X. laevis* (Kimelman et al., 1987). This is believed to reflect a requirement for speed and synchrony in early divisions (Hartwell and Weinert, 1989). In early *D. melanogaster* embryogenesis whilst the spindle integrity checkpoint clearly operates, the checkpoints controlling entry into mitosis do not. *D. melanogaster*, however, possesses an alternative mechanism to eliminate defective nuclei (Section 1.4.1.1).

Checkpoint controls are believed to delay mitotic progression by modulating the activity of the molecular oscillator which controls the cell cycle. The way this oscillator regulates mitosis is described in the next section.

1.2 How mitosis is regulated by the cell cycle control machinery

Cyclin dependent kinases (CDKs) are the core of the molecular oscillator that controls cell cycle progression in all eukaryotes. Homologues of the founder CDK, $p34^{cdc2}$ of *S. pombe*, control entry into mitosis in all eukaryotes. CDKs are also responsible for controlling other important transition points in the cell cycle such as the G1 to S phase transition. In yeasts the G1 to S transition is also controlled by $p34^{cdc2}$, but in vertebrates and possibly *D. melanogaster* (Section 1.5.4) related but distinct CDKs appear to be involved.

p34^{cdc2} is a highly stable protein whose enzymatic activity (phosphorylation of serine and threonine residues) is regulated by two mechanisms: positively by physical association with regulatory subunits known as cyclins; and either positively or negatively depending on the phosphorylation state of two critical residues. Cyclins are unstable proteins whose patterns of accumulation and loss are correlated with phases of the cell cycle (Evans et al., 1983). Cyclins can be distinguished on the basis of their sequence relationships and the transition points they are believed to influence. Cyclins A and B participate in the regulation of mitosis.

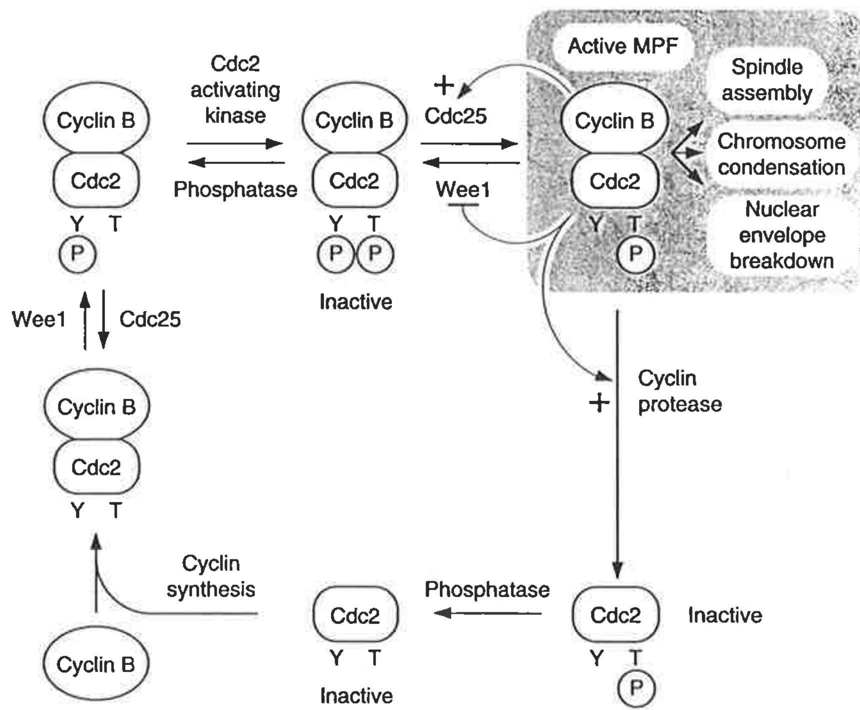
The current biochemical model for the regulation of mitosis by p34^{cdc2} (Figure 1.3) is a synthesis of results from many different organisms. Many mechanistic details are still to be determined, and the importance of particular regulatory steps in controlling cell cycle progression shows variation from species to species, and even within species across the life cycle (for example see Section 1.4.1.1).

Monomeric p34^{cdc2} is inactive and unphosphorylated (Figure 1.3). Association with cyclins A and B, which accumulate to a threshold level in G2, is a prerequisite for activation (Murray and Kirschner, 1989a). Formation of the complex induces phosphorylation at tyrosine 15 (nomenclature for human p34^{cdc2}) by kinases, first identified as the products of the *wee1* (Russell and Nurse, 1987b) and *mik1* (Lundgren et al., 1991) genes in *S. pombe*. Tyrosine 15 phosphorylation is inhibitory and dominant to cdc2 activating kinase (CAK) phosphorylation at threonine 161 (Solomon et al., 1992) by p40^{MO15} (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993), itself a CDK (Makela et al., 1994). Phosphorylation at these two residues produces inactive mitosis promoting factor (preMPF) which lacks protein kinase activity (Figure 1.3). Both phosphorylations are antagonised by the action of protein phosphatases, the former by homologues of *S. pombe* Cdc25 (Russell and Nurse, 1986; Moreno et al., 1990).

As the pool of preMPF increases a small amount of active MPF accumulates, dephosphorylated on tyrosine 15 (Figure 1.3). When the level of MPF reaches a threshold level it initiates an explosive process of activation that drives cells rapidly and irreversibly into mitosis. Wee1 is negatively regulated by yet another kinase Nim1, known from *S. pombe* (Russell and Nurse, 1987a; Coleman et al., 1993). MPF activates

Figure 1.3 Biochemical model of the mitotic oscillator (from Murray and Hunt, 1993).

The varied forms of the Cdc2-cyclin B complex, during the cell cycle, are shown. Also indicated are the enzymes that catalyse the modifications. The phosphorylation state of two critical residues corresponding to tyrosine 15 (left) and threonine 161 (right) in *S. pombe* are shown.



Active MPF
Spindle assembly
Chromosome condensation
Nuclear envelope breakdown
Cyclin B
Cdc2
Y
T
P
Cdc2 activating kinase
Phosphatase
Inactive
Cdc25
Wee1
Cyclin protease
Cyclin synthesis

Cdc25 establishing positive feedback (Solomon et al., 1990; Hoffman et al., 1993) (Figure 1.3), and may also activate Nim1 (Murray, 1993). In attaining full activity MPF also ensures its own demise by triggering the the cyclin degradation machinery which contributes to the exit from mitosis (see below).

MPF is believed to elicit its function by phosphorylating target proteins that mediate the events of mitosis, such as nuclear envelope breakdown, chromosome condensation and spindle formation. Although many proteins are phosphorylated in mitosis (Karsenti et al., 1987), and a plethora of proteins are substrates for MPF *in vitro*, very few proteins are known whose *in vivo* phosphorylation has consequences for mitotic progression. Probably the best characterised MPF substrates are the nuclear lamins, whose phosphorylation induces their depolymerisation (Hearld and McKeon, 1990), necessary but not sufficient for nuclear envelope breakdown (Nigg, 1993). MPF can modulate MT dynamics, and their nucleation by centrosomes in cell-free systems (Verde et al., 1990; Buendia et al., 1992), implicating MPF kinase activity in spindle formation. Furthermore, chromosome condensation is correlated with extensive phosphorylation of chromatin associated proteins (Reeves, 1992). However neither spindle formation nor chromosome condensation has yet been shown be dependent on the *in vivo* phosphorylation by MPF of a specific substrate.

The degradation of Cyclins A and B around the time of metaphase is necessary for inactivation of MPF, and coincides with onset of anaphase. Cyclin A is degraded during prometaphase, in advance of cyclin B, whose level drops precipitously at the metaphase-anaphase transition (Evans et al., 1983; Lehner and O'Farrell, 1990b; Whitfield et al., 1990). Cyclin degradation is believed to occur by ubiquitin mediated proteolysis, conferred by a motif in the N-terminal region, the "destruction box" (Glotzer et al., 1991). Demonstration that deletion of the cyclin B destruction box prevented inactivation of MPF and produced mitotic arrest in *X. laevis* (Murray et al., 1989), and *S. cerevisiae* (Ghiara et al., 1991) provided evidence for the hypothesis that cyclin destruction triggered anaphase onset by inactivating MPF (Murray and Kirschner, 1989b; Glotzer et al., 1991).

By contrast, recent findings from two studies indicate that initiation of anaphase requires neither cyclin degradation nor MPF inactivation. Addition of nondegradable, but

otherwise active cyclin B (same form as in Murray et al., 1989) to mitotically cycling *X. laevis* egg extracts prevents MPF inactivation but does not inhibit anaphase onset as determined by sister chromatid separation (Holloway et al., 1993). However other events of anaphase such as chromosome decondensation and spindle disassembly are blocked. The validity of these findings rests on interpretation of the arrested state as being anaphase. Earlier *in vivo* experiments (Murray et al., 1989) were reported to produce metaphase arrest.

Experiments with *S. cerevisiae* also provide evidence that mitotic cyclin degradation/MPF inactivation is not required for anaphase onset but functions in the final exit from mitosis (Surana et al., 1993). Mutants of *cdc15*, a protein kinase (Schweizer and Phillipsen, 1991), which arrest in telophase with disjoined chromosomes (Pringle and Hartwell, 1981), can undergo anaphase with high levels of MPF (Surana et al., 1993). Furthermore, overexpression of a B-type mitotic cyclin causes arrest in telophase not in metaphase.

If MPF inactivation is not a prerequisite for initiation of anaphase then what is the nature of the signal? Accumulating evidence supports the idea that chromosome disjunction is mediated by the same ubiquitin dependent proteolysis that degrades cyclin B. In *X. laevis* egg extracts sister chromatid separation is inhibited in a dose dependent manner by an N-terminal fragment of cyclin B that cannot activate MPF but is proposed to elicit its effect by competing, as a substrate for ubiquitination, with a hypothetical "anaphase trigger protein" (Holloway et al., 1993). Furthermore a mutant form of the N-terminal peptide that is not recognised by the ubiquitin conjugating system does not delay chromosome disjunction, while addition of the inhibitor, methylated ubiquitin does. Evidence also comes from mutations in genes encoding subunits of the 26S ATP/ubiquitin dependent protease in *S. pombe* (Gordon et al., 1993) and *S. cerevisiae* (Ghislain et al., 1993) that cause defective chromosome segregation. To date the hypothetical anaphase trigger protein targeted for proteolysis has not been identified in any organism.

Other lines of evidence point to a role for protein phosphatases in chromosome disjunction. The action of phosphatase(s) is a logical means of initiating disjunction as it

could simply counter the activity of a preexisting MPF substrate. Inhibition of protein phosphatases with okadaic acid inhibits disjunction in HeLa cells but permits cell cycle progression (Ghosh and Paweletz, 1992). Rat embryo fibroblast cells arrest at metaphase when injected with anti-protein phosphatase 1 (PP1) antibodies at the start of mitosis (Fernandez et al., 1992). Mutants in PP1 show defects in chromatid separation in *A. nidulans* (Doonan and Morris, 1989), *S. pombe* (Ohkura et al., 1989; Kinoshita et al., 1990), and *D. melanogaster* (Axton et al., 1990) (Section 1.5.3.2).

A transient rise in intracellular Ca^{2+} has also been invoked in the regulation of anaphase onset. Unfertilised *X. laevis* eggs arrested in metaphase II of meiosis enter anaphase when fertilisation triggers a cytoplasmic Ca^{2+} spike (Busa et al., 1985). Egg extracts have been used to develop *in vitro* systems that mimic many features of mitosis (Shamu and Murray, 1992; Holloway et al., 1993). Addition of Ca^{2+} to extracts with assembled metaphase spindles leads to chromosome segregation (with qualifications, see above), MPF inactivation, and passage into interphase. In sea urchin embryos sharp Ca^{2+} transients are correlated with cell cycle events including the metaphase-anaphase transition (Ciapa et al., 1994). Ca^{2+} levels may modulate the activity of calcium/calmodulin dependent protein kinases, substrates of which include a MAP whose phosphorylation at the metaphase-anaphase transition induces MT depolymerisation (Dinsmore and Sloboda, 1988).

The challenge remains to establish the relative contributions of, and the probable interactions between, proteolytic degradation, phosphorylation states, and Ca^{2+} levels in sister chromatid disjunction.

1.3 Genes involved in chromosome segregation in other well studied organisms

1.3.1 *Saccharomyces cerevisiae*

In a seminal achievement Hartwell and co-workers were the first to systematically identify genes involved in cell division. Many *c*ell *d*ivision *c*ycle (*cdc*) mutants were isolated in a genetic screen for temperature sensitive mutants whose uniform morphology

at the nonpermissive temperature indicated they were defective in cell cycle progression (Hartwell et al., 1970). These and further *cdc* mutants subsequently isolated have enabled the analysis of the *S. cerevisiae cdc2* homologue, *CDC28*, as well as genes required for budding, SPB duplication, DNA synthesis, spindle formation, and cytokinesis (reviewed in Hartwell, 1991).

Three *cdc* mutants, *cdc16*, *cdc23* and *cdc27* defective in mitosis appear to arrest in metaphase (Sikorski et al., 1990; Sikorski et al., 1991). Their wild-type genes encode proteins containing multiple tandem copies of an imperfect 34 amino acid "tetratricopeptide repeat" (TPR) present in, but not confined to, genes involved in mitosis (Sikorski et al., 1990). TPRs are proposed to function in intra- and intermolecular interactions with other TPRs (Sikorski et al., 1991; Sikorski et al., 1993) and all three proteins have now been shown to be part of a macromolecular complex (Lamb et al., 1994). Furthermore the products of *CDC16* and *CDC23* have been localised to the nucleus (Sikorski et al., 1993).

Other genetic screening strategies have identified mutants defective in chromosome disjunction, including two components of the CBF3 kinetochore complex (Section 1.1.3). Mutants in *esp1* arose from a screen for defects causing increased ploidy (Baum et al., 1988). *esp1* cells form irregular spindles and are grossly defective in chromosome segregation such that following cytokinesis the bulk of the DNA and both SPBs are found in one of the progeny cells, usually the daughter (McGrew et al., 1992). The hyperploid progeny are capable of undergoing at least one further round of DNA replication (McGrew et al., 1992) and spindle formation, accompanied by normal patterns of CDK activity and cyclin accumulation (Surana et al., 1993). This reinforces the notion that anaphase execution is independent of CDK destruction and cell cycle progression (Section 1.2).

1.3.2 *Schizosaccharomyces pombe*

Mutant screens have identified a number of genes required for chromosome segregation in *S. pombe* (Hirano et al., 1986). These include *dis1*, *dis2*, *dis3*, *sds1* and*sds2*, involved in the activity of protein phosphatase 1 (reviewed in Kinoshita et al., 1991) (Section 1.2). *nuc2* mutants arrest in a metaphase-like state with condensed

chromosomes and a short spindle (Hirano et al., 1988). p67^{nuc2} is an insoluble nuclear TPR protein (Hirano et al., 1990) that is the putative homologue of the *S. cerevisiae CDC27* product (Sikorski et al., 1991).

cut mutants are also defective in chromosome segregation but unlike *nuc2*$^{-}$ cytokinesis is not blocked (Hirano et al., 1986). Consequently the nuclei are often guillotined producing anucleate and hyperploid cells. Like *S. cerevisiae esp1* it appears that in *cut1* and *cut2* mutants DNA synthesis and SPB duplication can continue (Uzawa et al., 1990). Furthermore, *cut1* encodes a protein with a potential Ca^{2+} binding motif in a C-terminus with similarity to that of ESP1 (Uzawa et al., 1990). Genetic interactions suggest *cut1*, *cut2*, *cut4*, *cut8* and *cut10* may participate in the same, as yet uncharacterised, process (Uzawa et al., 1990; Murray and Hunt, 1993). *cut7* appears to be quite distinct, encoding a "bimC" KRP involved in SPB separation/spindle elongation (Hagan and Yanagida, 1990; Hagan and Yanagida, 1992) (Section 1.1.2). *cut9* which encodes a TPR protein is the apparent homologue of *S. cerevisiae CDC16* (Goebl and Yanagida, 1991).

1.3.3 *Aspergillus nidulans*

Conditional mutants identified in a genetic screen for defects in proliferation have been assigned to a number of classes (Morris, 1975). *nim* mutations (*n*ever *i*n *m*itosis), conceptually similar to *cdc* mutants, occur in genes so far shown to include homologues of *S. pombe cdc25*, *cdc13* (cyclin B) and *cdc2* (O'Connell et al., 1992; Osmani et al., 1994).

bim mutants (*b*locked *i*n *m*itosis) include the metaphase arrested *bimA*, a TPR protein gene functionally homologous to *S. cerevisiae CDC27* and *S. pombe nuc2* (Sikorski et al., 1991). The *bimA* product has been localised to the SPB implicating SPB function in chromatid disjunction (Mirabito and Morris, 1993). *bimB* is the putative homologue of *S. cerevisiae esp1* and *S. pombe cut1* and similarly, when defective, leads to failure in chromosome segregation but not DNA and SPB replication (May et al., 1992). *bimC* is the founder member of the distinct group of KRPs involved in SPB separation (Enos and Morris, 1990) (Section 1.1.2). The *bimE* product is not required for chromosome segregation *per se* but appears to be a unique negative regulator of mitosis that ensures

the completion of interphase before mitosis (Engle et al., 1990). Mutations in the protein phosphatase 1 gene *bimG* engender defects in chromosome segregation (Doonan and Morris, 1989) (Section 1.2).

1.4 Mitosis in *Drosophila melanogaster*

1.4.1 Mitosis in embryonic development

1.4.1.1 Syncytial divisions

The eggs of *D. melanogaster* complete oogenesis arrested in metaphase I of meiosis (Huettner, 1924). Egg maturation (completion of meiosis) is induced by ovulation (Doane, 1960), and in mated females is concurrent with fertilisation by a single stored sperm (Sonnenblick, 1950). The entire spermatozoan penetrates the egg whereupon all structures apart from its chromatin and a flagellar centriole pair are eventually lost (Karr, 1991). As the sperm nucleus chromatin decondenses one of the four female haploid nuclei approaches. The centrosome derived from the centriole pair divides and the two centrosomes orient to form the spindle poles for the first mitotic division (Huettner, 1924).

The maternal and paternal chromosomes undertake the first mitosis on a common spindle but in separate gonomeric groupings and do not achieve syngamy until telophase (Huettner, 1924). Postponement of pronuclear fusion until the end of cycle 1 is probably a product of the semi-closed form of mitosis observed for later divisions (Stafstrom and Staehelin, 1984) (Section 1.4.3).

Until prometaphase of cycle 1 the remaining three haploid products of female meiosis exhibit cell cycle coordination with the pronuclei then they terminally arrest (Rabinowitz, 1941). Their chromosomes remain condensed, their nuclear envelopes disperse, and eventually they fuse (Huettner, 1924). These polar bodies remain quiescent, except in the case of a class of maternal effect mutations (Section 1.5.1).

Apparently the act of fertilisation in some way initiates mitotic cycling that licenses the female pronucleus but not the polar bodies. Foe et al. (1993) have suggested that this

factor is the centrosome since the polar bodies may arrest at a metaphase like state in cycle 1, without a spindle, simply for want of centrosomes. However *abnormal spindle* (*asp*) females can produce eggs devoid of DNA that do not sustain proliferation of the male pronucleus following fertilisation (Gonzalez et al., 1990), so some factor associated with the female pronucleus must also be involved.

The centrosome in *D. melanogaster* is organised by paternally supplied centrioles, derived from the sperm tail basal body. It is important to distinguish between centrosomes and centrioles when considering reports of centrosomes arising *de novo* as in unfertilised embryos of *giant nuclei* or *asp* mothers (Freeman and Glover, 1987; Gonzalez et al., 1990), or in rare cases of parthenogenesis (Carson, 1967). Conceivably centrosomal/spindle pole structures may form that are not organised by centrioles. Indeed an acentriolar *D. melanogaster* cell line can still perform mitosis (Debec and Montmory, 1992).

Following completion of cycle 1 the ensuing 12 cycles consist of a series of rapid, nearly synchronous nuclear divisions in a syncytium. These divisions consist of alternating rounds of S and M phases with no discernible gap phases. Until cycle 7 the nuclei occupy the interior of the egg. Then in cycles 8 and 9 the majority of the nuclei migrate towards the egg surface in two discrete steps (Foe et al., 1993) in a MT dependent manner (Raff and Glover, 1989). During each of these steps some nuclei fail to migrate and regress to the interior of the embryo. The approximately 200 vitellophage nuclei that result, cease dividing after cycle 10, lose their centrosomes, and become polyploid.

The first nuclei to reach the egg surface arrive at the posterior pole in cycle 9. The remainder reach the periphery of the egg in cycle 10 and become distributed in an evenly spaced monolayer, the syncytial blastoderm configuration. At this time the nuclei at the posterior pole cellularise to form the pole cells, the progenitors of the germ line.

Mitotic cycles 2 to 9 are extremely rapid, but from cycle 10 onwards there is a progressive lengthening of cycle times that coincides with increased levels of zygotic transcription. At 24° cycles 2 through 9 take on average 8.4-8.8 minutes (Rabinowitz, 1941; Foe et al., 1993), whilst cycles 10 to 13 take 8.8, 10, 13 and 16-17.5 minutes

respectively at 25° (Foe, 1989; Foe et al., 1993). This occurs independent of transcription (Edgar et al., 1986) and is not contingent on any genomic region (Merrill et al., 1988; Wieschaus and Sweeton, 1988). By experimental manipulation of nuclear density (Edgar et al., 1986) and ploidy (Zalokar et al., 1975; Edgar et al., 1986) increasing cycle length has been shown to be correlated with increased nuclear:cytoplasmic ratio. However the factor(s) which elicit this phenomenon are unknown.

The syncytial blastoderm cycles 10 through 13 are notable for undergoing mitosis "metachronously" (Foe and Alberts, 1983). Typically, mitosis initiates in nuclei at both embryonic poles and propagates in the manner of a wave towards the equator. It has been estimated that this mitotic wave takes as little as 30 seconds to traverse from pole to equator (Foe and Alberts, 1983). Whilst nuclei at the egg termini are the first to begin (and conclude) mitosis, during any cycle each nucleus has about the same cycle length.

Foe et al (1993) have suggested that this mitotic wave is propagated by diffusion of an autocatalytic mitotic activator throughout the common cytoplasm of the syncytium. They believe that MPF is a candidate for such an activator. Certainly p34*cdc2*, which shows little fluctuation in activity in early cycles, becomes subject to greater oscillations in kinase activity at the time of the metachronous divisions (Edgar et al., 1994). However the inhibitory phosphorylation at tyrosine 15, whose dephosphorylation by Cdc25 is subject to positive feedback in other systems (Section 1.2), cannot be detected in *D. melanogaster* at this stage (Edgar et al., 1994). Rather, cycles 10 to 13 have been shown to be timed by the accumulation of cyclins (Edgar et al., 1994), a form of regulation for which positive feedback has not been demonstrated and is difficult to envisage.

Despite the diminished degree of cell cycle regulation in syncytial blastoderm embryos the spindle assembly checkpoint clearly operates. Treatment with microtubule inhibitors arrests mitosis with chromosomes in a metaphase like configuration (Zalokar and Erk, 1976; Foe and Alberts, 1983). Consistent with the global action of a checkpoint all subsequent nuclear and cytoplasmic events are blocked (Foe et al., 1993). In both syncytial embryos (Foe et al., 1993) and in larval neuroblasts (Whitfield et al., 1990) the arrest is correlated with elevated, metaphase like levels of cyclin B (Section 1.5.4).

By contrast the checkpoint mechanisms that block entry into mitosis as a result of DNA damage or underreplication appear not to operate in syncytial embryos. Embryos that have been X-irradiated (Schneider-Minder, 1966) or have had DNA replication blocked with aphidicolin (Raff and Glover, 1988; Raff and Glover, 1989) continue nuclear and cytoplasmic events unhindered for several cycles. It appears that these checkpoints may commence operating following cellularisation (Foe et al., 1993).

In the absence of these checkpoints syncytial blastoderm embryos employ a different mechanism to ensure that the progeny of abnormal nuclear divisions do not contribute to the differentiated cellular complement. Abnormal nuclei that form, when DNA replication is blocked (Raff and Glover, 1988; Yasuda et al., 1991), in mutants with colliding nuclei (Sullivan et al., 1990; Postner et al., 1992; Fogarty et al., 1994), when topo II is inhibited (Buchenau et al., 1993), as a consequence of delay in metaphase alignment (Sullivan et al., 1993) or in rare cases of defective division in wild-type embryos (Minden et al., 1989), lose their attachment to the centrosome (see frontpiece) and sink into the interior of the embryo. The mechanism of this detachment is unknown.

The adaptability of this system for mitotic fidelity is indicated by the detection of compensatory divisions in surrounding unaffected nuclei (Yasuda et al., 1991; Buchenau et al., 1993) possibly stimulated by the decreased nuclear:cytoplasmic ratio (see above). This may explain the report that some *daughterless-abo-like* mutant embryos have regions of increased nuclear density, and a significant proportion can develop to adulthood (Sullivan et al., 1990).

The syncytial divisions halt after 13 cycles via an unknown mechanism, proposed to be zygotically activated (O'Farrell et al., 1989). Maternal mRNA is sufficient for all 13 divisions as the RNA synthesis inhibitor α-amanitin blocks all subsequent development if injected into embryos at or before cycle 13 (Edgar et al., 1986). Zygotic transcription first detected during syncytial blastoderm divisions may produce a factor, a ribonuclease has been suggested (O'Farrell et al., 1989), which inactivates a product required for mitosis. *string,* the *D. melanogaster* homologue of the *S. pombe cdc25* gene, is a credible candidate for such targeted inactivation because its maternal mRNA is abruptly degraded following

mitosis 13 (Edgar and O'Farrell, 1989), and because of its demonstrated role in regulating mitosis in subsequent cycles (see below).

1.4.1.2 Post-cellularisation divisions

A profound transition occurs at the commencement of cycle 14, characterised by a number of coordinated phenomena. Zygotic transcription, first detectable in cycle 10, increases dramatically in interphase 14 and it is these newly synthesised transcripts which regulate most of these processes. Cycle 14 is the first cycle with an extended interphase, resulting from the introduction of a G2 phase. During the first half of interphase 14 (Foe and Alberts, 1983) membranes form between the blastoderm nuclei generating an epithelial monolayer of blastoderm cells.

The cellular blastoderm is shortlived, for gastrulation begins immediately following cellularisation transforming the epithelial monolayer into the multilayered tissues of a larva. Groups of cells invaginate and the embryo elongates in a process termed "germ band extension". As a consequence of its confinement within the chorion, the embryo folds over on itself propelling the pole cells dorsally, anteriorally and ultimately internalising them.

Simultaneous with germ band extension mitosis is initiated in a complex and invariant pattern of domains that have been shown to coincide with primordia of some larval organs and tissues (Foe, 1989). The cycle 14 mitotic domains represent the earliest manifestation of the commitment of cells to a specific developmental fate. In a seminal achievement these have been documented in remarkable detail by Victoria Foe (Foe, 1989).

Cells within cycle 14 mitotic domains enter mitosis in close synchrony with each other but temporally distinct from cells in other mitotic domains. As all cells complete cycle 14 DNA replication at the same time, the order in which the mitotic domains are activated is dependent on the duration of the G2 phase. This order is reflected in the designation of mitotic domains (Foe, 1989). Thus domains 1 and 25 enter mitosis 70 and 115 minutes, respectively, after completing mitosis 13. Exceptions to this pattern of cycle

14 divisions are nonproliferating cells, of the amnioserosa (domain A) which arrest at G2 of cycle 14, and possibly a region of the presumptive head (domain B) (Foe, 1989).

The initiation of mitosis in the cycle 14 domains appears to be regulated by expression of *string*. During cycle 14 (and the ensuing 2 cycles) *string* is expressed in a spatially and temporally dynamic pattern that precisely anticipates, by 15 to 35 minutes, the pattern of mitotic domains (Edgar and O'Farrell, 1989). Moreover ectopic expression of *string* using a heat-shock promoter is sufficient to drive all G2 cells rapidly into mitosis (Edgar and O'Farrell, 1990). As *string* mutants are blocked in G2 of cycle 14, *string* expression is both sufficient and necessary for mitotic initiation in cellular divisions (Edgar and O'Farrell, 1990).

If *string* controls the complex spatiotemporal pattern of proliferation in cycles 14-16 how is its expression regulated? The *string* locus is known to contain extensive arrays of *cis*-acting regulatory elements that confer different subsets of the cycle 14 mitotic domains (B. Patterson and R. Saint, pers. comm.; Foe et al., 1993). It is proposed (Edgar and O'Farrell, 1989) that the timing and location of *string* expression is realised by the integration of information from combinations of patterning genes expressed during embryogenesis, such as those involved in segment formation and identity. The experimental confirmation of this hypothesis, currently underway, involves the dissection of promoter elements residing in tens of kilobases of flanking untranslated sequence (B. Patterson and R. Saint, pers. comm.; Foe et al., 1993).

Despite the abundance of its maternal mRNA and protein in syncytial embryos the regulatory role of *string* , if any, in cycles 1 to 13 remains enigmatic. Cell cycle dependent phosphorylation of string, believed to control its activity, is detectable from cycle 5 but no corresponding changes in cdc2 tyrosine phosphorylation are apparent. Instead fluctuations in cdc2 activity in syncytial embryos are correlated with cyclin levels (Edgar et al., 1994).

Following cycle 14 most cells undergo two further division cycles. Cycle 15 and 16 divisions, like those in cycle 14, occur in domains, most of which represent subdivisions of cycle 14 domains (Foe et al., 1993), and are similarly preempted by *string* expression (Edgar and O'Farrell, 1989).

Certain embryonic cell lineages continue proliferating beyond cycle 16. Neuroblasts of the Central Nervous System (CNS) which arise in cycle 14 by delamination from the ventral ectoderm are capable of up to 9 asymmetric divisions in embryogenesis (Hartenstein et al., 1987). Peripheral Nervous System (PNS) cells, derived from precursor cells which separate from the dorsal-lateral ectoderm after cycle 15 (Hartenstein and Campos-Ortega, 1985), undergo up to 3 rounds of proliferation (Bodmer et al., 1989; Ghysen and O'Kane, 1989). In addition a subset of dorsal epidermal cells in thoracic segments 1 and 2 undergo cycle 17 (Bate and Martinez Arias, 1991) to form part of the tracheal system (Knoblich et al., 1994).

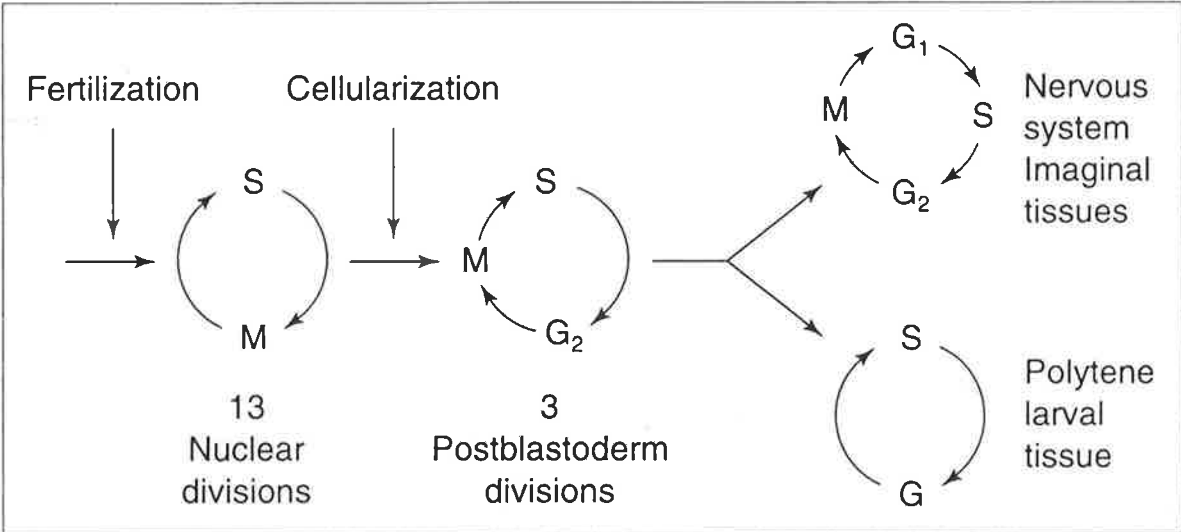
Proliferation subsequent to cycle 16 is characterised by a further significant transition, the addition of a G1 phase to the cell cycle (Figure 1.4). For cycles 1 to 15 initiation of DNA replication is constitutively coupled to mitosis (Edgar et al., 1986; Edgar and O'Farrell, 1990) (Figure 1.4). Even additional cellular mitoses, driven by ectopic *string* expression, are followed by obligate S phases (Edgar and O'Farrell, 1990). By contrast, normal cycle 16 mitoses are not followed by an immediate round of replication (Edgar and O'Farrell, 1990), and most cells enter G1 (Edgar and O'Farrell, 1990; Smith and Orr-Weaver, 1991), where many become terminally arrested.

While regulation of *string* expression controls progression from G2 from cycle 14 onwards it appears that, following the introduction of a G1 phase, entry into S phase is regulated by expression of the G1 cyclin *DmcycE* (Richardson et al., 1993; Knoblich et al., 1994). In non-neural cells *DmcycE*, whose expression is constitutive in during cycles 14 to 16 (Richardson et al., 1993), is down-regulated just prior to G1 arrest in cycle 17 (Knoblich et al., 1994). Furthermore ectopic expression demonstrates that *DmcycE* is sufficient for entry into S phase 17 in G1 arrested cells (Knoblich et al., 1994).

By 7.5 hours after egg deposition (AED) cycle 16 concludes in non-neural cells and the embryo undergoes germ band retraction to assume a larval morphology. Although no longer capable of mitosis many cell types continue DNA replication post cycle 16 without karyokinesis to become polytene (polyploid) (Smith and Orr-Weaver, 1991). These endoreplicative cycles begin 8 hours AED, in progenitors of the larval salivary glands, midgut, hindgut, fat body, and Malpighian tubules, in a defined spatiotemporal order (Smith

Figure 1.4 Summary of the types of cell cycle during development of *D. melanogaster* (from Orr-Weaver, 1994).

The first 13 cycles occur, without discernible gap phases, in a syncytium. A G2 phase is added to the cell cycle for the subsequent 3 cellular divisions. Following cycle 16 a G1 phase is observed in proliferation of neural tissues, whilst many other larval tissues enter an endo cell cycle in which mitosis does not occur, leading to polyteny.


Fertilization
Cellularization
S
M
13
Nuclear
divisions
S
M
G2
3
Postblastoderm
divisions
G1
M
S
G2
Nervous
system
Imaginal
tissues
S
G
Polytene
larval
tissue

and Orr-Weaver, 1991), analogous to the cycle 14 mitotic domains. "Endocycles" represent a modified form of the normal cell cycle consisting solely of alternating S and G phases (Figure 1.4), but the nature of their regulation is unknown. Only one regulatory factor, *DmcycE*, has been shown to be required for endoreplication (Knoblich et al., 1994).

The pole cells, which were set aside early in embryogenesis, follow their own proliferative agenda. They divided a maximum of two times following cellularisation in cycle 10 (Technau and Campos-Ortega, 1986), are propelled internally by gastrulation and remain quiescent until incorporated into the presumptive gonads. Late in embryogenesis, at about 16 hours AED, they divide a further one or two times (Sonnenblick, 1950).

1.4.2 Mitosis in postembryonic development

Two principal types of cells remain diploid and mitotically active after hatching. Persistent embryonic CNS neuroblasts of the thoracic segments, which were arrested in G1, commence proliferation by 36 hours after hatching, continue dividing throughout larval life and metamorphose into adult neurons (Truman and Bate, 1988).

The tissues of the adult (the imago) consist almost entirely of the progeny of discs or nests of cells that are sequestered from embryonic lineages. The imaginal cells remain quiescent until proliferation is initiated in different discs at varying stages of larval development, beginning during the first larval instar (Madhavan and Schneiderman, 1977) and ceasing in pupation.

1.4.3 The nuclear and cytoskeletal organisation of mitosis

Studies of the subcellular structures of mitosis have exploited the configuration of the syncytial blastoderm. In cycles 10 and 11 numerous, relatively large spindles undergoing metachronous divisions lie in a thin layer just below the surface of the embryo, making them accessible to visualisation and manipulation. These studies have revealed the cyclical patterns of MT and actin cytoskeletal dynamics, as well as the nuclear and centrosomal cycles (Foe and Alberts, 1983; Stafstrom and Staehelin, 1984; Karr and Alberts, 1986; Warn and Warn, 1986; Warn et al., 1987; Kellogg et al., 1988).

In particular, evidence is accumulating for the pivotal role of the centrosome in coordinating the nuclear and cytoskeletal aspects of the cycle. Apart from their well established role in organising the mitotic spindle, the MT arrays that centrosomes nucleate are essential for nuclear migration, organising the cytoplasm and plasma membrane, cellularisation and cytokinesis. Centrosomes are capable of performing most of these functions independent of nuclei (Raff and Glover, 1988; Raff and Glover, 1989; Yasuda et al., 1991). If the DNA synthesis inhibitor aphidicolin is injected into syncytial embryos at cycle 7 or 8 nuclear migration is blocked but the centrosomes continue to replicate and migrate to the embryo surface, where they organise the cortical cytoskeleton and initiate pole cell formation (Raff and Glover, 1989). Furthermore, cyclical patterns of chromosome condensation and decondensation, and reorganisation of the nuclear envelope continue in aphidicolin treated embryos (Raff and Glover, 1988). It therefore appears that the nuclear aspects of division cycles can operate independently of the cytoplasmic events organised by the centrosome, synchrony being dictated by the cell cycle machinery.

The duration of each phase of mitosis in cycle 10 has been quantified. Foe and Alberts (1983) estimated the phase lengths by extrapolating from the proportion of embryos counted in each stage. (Kellogg et al., 1988) timed the phases by observing the characteristic changes in MT organisation in embryos injected with fluorescent derivatives of tubulin. Incorporating the earlier data (Foe and Alberts, 1983) Kellogg et al. (1988) report lengths of 0.4, 1.6, 1.6, 1.0, and 0.4 minutes for prophase, prometaphase, metaphase, anaphase and telophase of cycle 10, with the ensuing interphase of cycle 11 taking 5.5 minutes.

In a typical nuclear cycle in the syncytial blastoderm a pair of centrosomes adjacent to each other on the cortical surface of the nucleus migrate to opposite sides of the nuclear envelope in *interphase* (Warn and Warn, 1986; Kellogg et al., 1988). Centrosomal migration is believed to be mediated by antiparallel interaction (Foe et al., 1993) (Section 1.1.2) between MTs that emanate from each centrosome and traverse the surface of the nuclear membrane (Warn and Warn, 1986; Kellogg et al., 1988). Concomitant with centrosomal migration the nuclei swell and chromatin decondenses indicative of DNA replication.

In *prophase* chromosome condensation is initiated at multiple sites of attachment to the nuclear envelope, and propagates along the chromosome arms (Hiraoka et al., 1989). MT dynamics undergo a major transition which converts the interphase organisation of long MTs to a dense array of short labile MTs emanating from each centrosome (Warn et al., 1987; Kellogg et al., 1988). Possibly as a consequence of the impact of elongating MTs in this region, the section of the nuclear envelope nearest the centrosome begins to break down (Stafstrom and Staehelin, 1984).

During *prometaphase* the semi-closed form of mitosis in *D. melanogaster* becomes apparent. The condensed chromosomes detach from the nuclear envelope (Hiraoka et al., 1990) which remains intact except in the vicinity of the spindle poles (Stafstrom and Staehelin, 1984). A second morphologically similar envelope forms around the remnant nuclear envelope, collectively these are termed the spindle envelope (Stafstrom and Staehelin, 1984). In spite of the incomplete breakdown of the nuclear envelope, nuclear lamins, which form a latticework which underpins the nuclear envelope and whose dissociation is requisite for open mitoses, are disassembled (Fuchs et al., 1983) as are nuclear pore complexes (Stafstrom and Staehelin, 1984). MTs emanating from the centrosomes permeate the spindle envelope to form the spindle while some MTs extend around the envelope's perimeter (Warn et al., 1987; Kellogg et al., 1988). Spindle MTs interact with kinetochores, chromosome arms, and with MTs nucleated by the other spindle pole. As a result the condensed chromosomes become aligned on the *metaphase* plate. It is assumed that MT dynamics and/or molecular motors contribute to metaphase congression (and anaphase disjunction) in the same manner as in other organisms (Section 1.1.5) but little is known of their precise contributions in *D. melanogaster*.

Anaphase is marked by a reversal of the pattern of MT dynamics established in prophase, so that MT stability increases. Indicative is the appearance of prominent astral MTs (Warn et al., 1987; Kellogg et al., 1988). The sister chromatids separate and move towards the spindle poles as the kinetochore MTs shorten (anaphase A). Simultaneously the spindle elongates, increasing the distance between the spindle poles, and further disjoining the chromatids (anaphase B). Conceivably the force generation for anaphase B could be conferred by interactions between spindle overlap MTs, or by interactions of the

astral MTs with the asters of neighbouring spindles or some other potential anchor (Section 1.1.5).

In *telophase* the daughter nuclei continue to move apart as their chromosomes begin decondensing. The spindle envelope dissociates to form the monolaminate nuclear envelope coalescing last around the telomeric ends of the chromosomes, distal to the centrosomes (Fuchs et al., 1983). As the spindle overlap MTs are lost an array of MTs, known as the "midbody" (Warn et al., 1987) or "interzonal MTs" (Kellogg et al., 1988), persists late into telophase spanning the space between daughter nuclei. Midbody MTs are not nucleated by the centrosome, but run from nucleus to nucleus (Warn and Warn, 1986), raising questions as to their role (see *grapes* in Section 1.5.1) and how they are organised. As telophase proceeds the nuclei undergo a rotation of 90° so that they reorientate from the plane of division tangential to the embryo surface till the centrosomes are on the cortical side of the nucleus. Also in telophase the duplication and separation of centrosomes, which commenced in metaphase with the separation of the centrioles in each centrosome, is completed (Callaini and Riparbelli, 1990). The centrioles then duplicate in the ensuing interphase and prophase (Callaini and Riparbelli, 1990).

The addition of the process of cytokinesis (not considered in detail here), after completion of karyokinesis, is one of the few ways in which cellular mitoses differ from the general pattern of mitosis described above. The spindle envelope has been observed in these divisions (cited in Stafstrom and Staehelin, 1984). However centrosome migration occurs later, in prophase, in cellular divisions (Callaini and Riparbelli, 1990).

1.5 Genes involved in mitosis in *D. melanogaster*

By exploiting its amenability to genetic analysis *D. melanogaster* has become the best understood multicellular organism with respect to mitosis. A plethora of genes affecting cell proliferation have been indentified in screens for mutants employing various strategies, but few have received more than superficial examination. Many descriptions of phenotype are based on single alleles, without analysis of hemizygous interactions with deficiencies or other alleles to determine the precise nature of the defect. I will document

genes whose mutant phenotype has been characterised in detail, and concentrate on those in which the gene has been cloned and analysis of its product has provided clues to its precise function in mitosis. These genes are shown in Figure 1.5 in relation to their proposed role in molecular and subcellular events of mitosis.

The *D. melanogaster* embryo is richly endowed by its mother with products required for mitosis. The three classes of mutants defective in proliferation commonly distinguished, maternal effect, late larval lethal and embryonic lethal, loosely reflect the stage to which the maternal complement normally persists, and when zygotic transcription usually commences. These categories are not mutually exclusive. Mutants that are late larval lethal when null, often manifest as maternal effect with weaker alleles. In addition to their lethality in the F_2 generation some maternal effect mutants exhibit (nonlethal) defects in mitosis in the CNS neuroblasts of the preceding generation.

An additional class of mutants discussed here are those which affect meiosis as well as mitosis.

1.5.1 Maternal effect genes

The genes defined by the maternal effect class of mutants are generally those whose products are required for the very early (syncytial) divisions. Homozygous mutant individuals develop normally, using products derived from their (heterozygous) mothers. They are unable, however, to supply their progeny with these products and are thus sterile. Foe et al. (1993) list more than 60 mutations whose mitotic phenotype in syncytial embryos is evident as a maternal effect.

Three interacting genes have been identified which act to repress DNA replication in unfertilised eggs and to negatively regulate S-phase in early cleavage divisions. Females mutant in *giant nuclei* (*gnu*) (Freeman et al., 1986; Freeman and Glover, 1987) *plutonium* (*plu*) and *pan gu* (Shamanski and Orr-Weaver, 1991) produce unfertilised eggs which have undergone extensive DNA synthesis, and fertilised eggs in which S-phase is uncoupled from mitosis resulting in giant polyploid nuclei. The *plu* gene encodes a small basic protein with two ankyrin repeats, a domain implicated in protein-protein interactions

(Axton et al., 1994). Another maternal effect mutation *supernova* (*sun*) (Underwood et al., 1990) has an analogous phenotype.

Embryos produced by *fs(1)Ya* mothers are arrested in the first, gonomeric, embryonic mitosis (Lin and Wolfner, 1991). *fs(1)Ya* , encodes a 708 residue highly basic protein (Lin and Wolfner, 1989) which localises to the nuclear lamina in a cell cycle dependent manner (Lin and Wolfner, 1991; Lopez et al., 1994).

Phenotypic analysis of *aurora* (Glover, 1989) and *daughterless-abo-like* (*dal*) (Sullivan et al., 1990) suggest they are involved in the separation of centrosomes. *aurora* encodes a serine/threonine protein kinase (D. Glover, pers. comm.).

The phenotypic consequences on embryos of mutation in *abnormal spindle* (*asp*)are complex, including defects in mitotic synchrony and spindle formation (Gonzalez et al., 1990). *asp* also affects meiosis and post embryonic mitoses (Ripoll et al., 1985). *asp* encodes a highly basic 205kD "pioneer" protein (D. Glover, pers. comm.).

Mutation of *grapes* causes failure of midbody/interzonal microtubule formation in the syncytial blastoderm, and consequently to collision of daughter nuclei (Fogarty et al., 1994).

The *lodestar* defect is tangling and chromatin breakage in anaphase. *lodestar* encodes a 974 aa NTP binding protein, possibly a helicase, that shuttles between the nucleus and cytoplasm in mitosis and interphase respectively (Girdham and Glover, 1991). *quartet* (*qrt*) exhibits complete failure of chromosome disjunction without impediment of cell cycle progression (Zahner and Cheney, 1990).

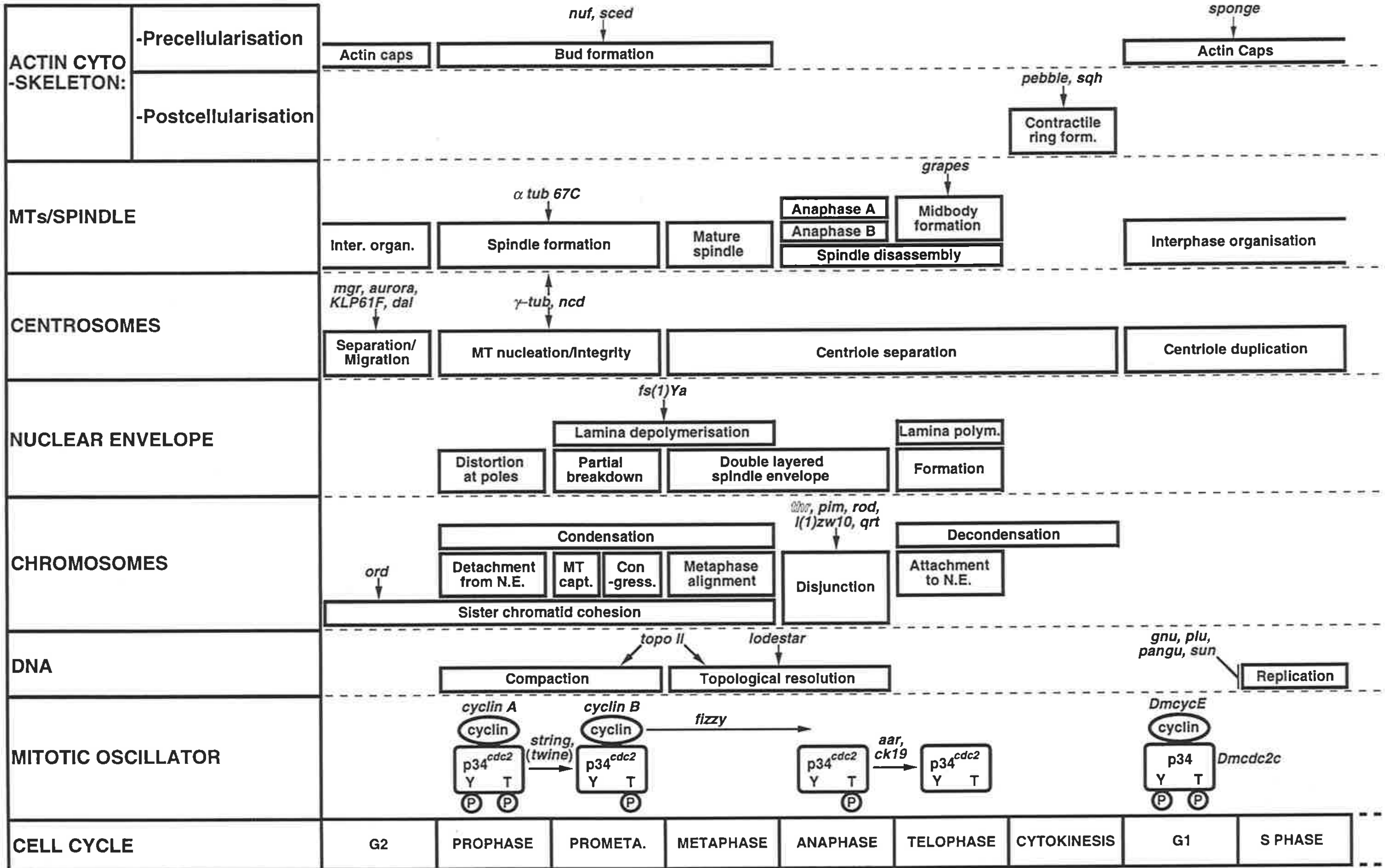
Precise function is difficult to assign to *abnormal chromatin* (*abc*)(Vessey et al., 1991) and a distinct *supernova* (*spno*) (Webster et al., 1992), as both exhibit extreme pleiotropic effects from the first syncytial divisions.

1.5.2 Meiotic mutants

While some mutants that affect gametogenesis are exclusively meiotic, evidence for shared mechanisms in meiosis and mitosis comes from the incidence of mutants defective in both processes (see *asp* in Section 1.5.1).

Figure 1.5 Flow diagram of events in various molecular and subcellular pathways during the cell cycle of *D. melanogaster*.

The role of various essential genes in these processes, as implied by their mutant phenotype, is indicated. Cell cycle, and mitotic oscillator shown in late embryogenesis modes, although some genes shown may only act in syncytial divisions. *gnu*, *plu*, *pangu* and *sun* are believed to act as inhibitors of DNA replication. *cyclin A* and *B* are shown at the stage at which they are believed to first associate with $p34^{cdc2}$. Because of the pleiotropic effects of their mutation no attempt to attribute function to *polo, abc*, *supernova* (*spno*), *dhd* or *asp* is made. In addition *aar*, and *ck19* phosphatases probably have a broad range of substrates. *nuf* (*nuclear-fallout*), *sced* (*scrambled*), *sponge* and *sqh* (*spaghetti-squash*) are not described in the text.



ACTIN CYTO -SKELETON:
-Precellularisation
-Postcellularisation
MTs/SPINDLE
CENTROSOMES
NUCLEAR ENVELOPE
CHROMOSOMES
DNA
MITOTIC OSCILLATOR
CELL CYCLE
nuf, sced
sponge
Actin caps
Bud formation
Actin Caps
pebble, sqh
Contractile ring form.
grapes
α tub 67C
Anaphase A
Anaphase B
Midbody formation
Inter. organ.
Spindle formation
Mature spindle
Spindle disassembly
Interphase organisation
mgr, aurora, KLP61F, dal
γ-tub, ncd
Separation/ Migration
MT nucleation/Integrity
Centriole separation
Centriole duplication
fs(1)Ya
Lamina depolymerisation
Lamina polym.
Distortion at poles
Partial breakdown
Double layered spindle envelope
Formation
thr, pim, rod, l(1)zw10, qrt
Condensation
Decondensation
ord
Detachment from N.E.
MT capt.
Con -gress.
Metaphase alignment
Disjunction
Attachment to N.E.
Sister chromatid cohesion
topo II
lodestar
gnu, plu, pangu, sun
Compaction
Topological resolution
Replication
cyclin A
cyclin B
fizzy
DmcycE
cyclin
string, (twine)
aar, ck19
p34cdc2
p34
Y T
P
Dmcdc2c
G2
PROPHASE
PROMETA.
METAPHASE
ANAPHASE
TELOPHASE
CYTOKINESIS
G1
S PHASE

Mutants in *orientation disruptor* (*ord*) cause premature sister chromatid disjunction (i.e. intra- not interchromosomal) in meiosis in the male and female germline (Miyazaki and Orr-Weaver, 1992). Although sister chromatid cohesion is also defective in mitosis in male gametogenesis *ord* is evidently not required for somatic mitosis.

By contrast, a small group of mutants defective in meiosis also derange embryonic mitoses. Unlike most tubulin isoforms which are ubiquitously expressed and can be functionally substituted for by other isoforms αTUB67C is essential for nuclear division in the oogenesis and embryogenesis (Matthews et al., 1993). 90% of eggs laid by *deadhead* (*dhd*) mothers are nonviable due to defective meiosis and the remainder show asynchrony and polyploidy in embryonic mitosis (Salz et al., 1994). *dhd* encodes a 107 aa homologue of thioredoxins, capable of protein disulphide bond cleavage, implicating yet another form of posttranslational modification in mitotic regulation.

Two genes encoding KRP motors required for meiosis also function in mitosis. *claret nondisjunctional* (*cand*) mutants of the *non-claret disjunctional* (*ncd*) locus are defective in chromosome segregation in female meiosis, and embryonic mitosis up to cycle 5, as a consequence of severe disruption of the spindle (Hatsumi and Endow, 1992). *ncd* encodes a minus end directed KRP (Endow et al., 1990; McDonald et al., 1990) localised to the mitotic spindle poles and proposed to function in their maintainence (Endow et al., 1994). By contrast loss-of-function alleles of *no distributive disjunction* (*nod*) which disrupt chromosome segregation in meiosis do not appear to affect mitosis (Zhang et al., 1990). However the *nod* transcript is detectable throughout development suggesting a function in mitosis (Zhang et al., 1990) that may be obscured by redundancy (Section 1.1.5).

1.5.3 Zygotically regulated genes

1.5.3.1 The influence of the maternal contribution on the time of onset of the zygotic phenotype

Following the approaches pioneered by Hartwell and coworkers in *S. cerevisiae* the genetic tractability of *D. melanogaster* has made it a focus for the identification of essential

cell cycle genes in multicellular organisms. However initial attempts to define conditional or embryonic lethal mutants in genes involved in control of cell proliferation were largely unsuccessful. The conceptual breakthrough came from consideration of *D. melanogaster* ontogeny (Baker et al., 1982). Following the completion of embryogenesis growth throughout larval life is accomplished by increases in cell size not number. Proliferation in larval development occurs in neural and imaginal tissues that are the progenitors of adult structures but are dispensible for larval viability (Shearn et al., 1971). If most products required for embryonic cell division are provided as maternal mRNA and/or protein then a zygotic requirement for gene expression would not be manifest until the formative period of the next life cycle stage requiring significant cellular proliferation, the adult. Accordingly, progeny homozygous mutant for an essential cell division gene would die at the larval/pupal transition from defects in imaginal tissue proliferation.

A prediction of the hypothesis of Baker, Gatti and coworkers is the occurrence, in collections of "late larval lethals", of mutants in genes for essential proliferative functions. Indeed cytological examination of CNS neuroblasts in 59 late larval lethals revealed 30 were defective in various aspects of mitotic chromsome behaviour (Gatti and Baker, 1989). Some of the better characterised of these mutants are amongst those described below.

1.5.3.2 Late larval lethals

The phenotypic consequences of mutations in the *merry-go-round* (*mgr*) and *KLP61F* (Heck et al., 1993) (Section 1.1.2) genes are consistent with defects in the separation of centrosomes. *mgr* mutants exhibit rings of condensed chromosomes around monopolar spindles in larval neuroblasts and also perturb meiosis (Sunkel and Glover, 1988).

Both *rough deal* (*rod*) (Karess and Glover, 1989) and *l(1)zw10* (Williams et al., 1992) are defective in chromosome segregation. *l(1)zw10* encodes a 721aa "pioneer" protein, zw10, with a cell cycle dependent pattern of localisation. In prometaphase zw10 migrates from the cytoplasm to the spindle/nucleus, and associates in metaphase and anaphase with structures coincident with kMTs and kinetochores respectively (Williams

et al., 1992). Unlike those of wild-type, chromosomes in *l(1)zw10* mutant cells can undergo "precocious sister chromatid separation" (PSCS) when mitotically arrested by either colchicine or taxol (Williams and Goldberg, 1994). Furthermore PSCS occurs in the presence of high levels of cyclin B. *l(1)zw10* is proposed to be involved in the spindle integrity feedback pathway (Williams and Goldberg, 1994). The pattern of localisation of zw10 is significantly altered in two other mutants defective in chromosome segregation, *rod* and *abnormal anaphase resolution* (this section), suggesting interaction with the products of these genes.

Lesions in both protein phosphatase 1 and 2A have been identified in mutants with defects in chromosome segregation. In *abnormal anaphase resolution* (*aar*) mutants sister chromatids separate but are defective in polewards movement (Gomes et al., 1993; Mayer-Jaekel et al., 1993). *aar* encodes the regulatory subunit of protein phosphatase 2A (Mayer-Jaekel et al., 1993), which is also defective in the more severely compromised, independently isolated allelic mutant *twins* (Uemura et al., 1993). Mutants of *ck19* (*PP1 87B*), encoding a protein phosphatase 1 isoenzyme, are defective in a number of events late in mitosis including chromosome disjunction (Axton et al., 1990).

polo encodes a member of a conserved family of (*S. cerevisiae*) *CDC5*-like serine-threonine protein kinases implicated in the regulation of late mitotic (and meiotic) events (Golsteyn et al., 1994). *polo* mutants exhibit pleiotropic effects in mitosis and meiosis (Sunkel and Glover, 1988; Llamazares et al., 1991). Polo kinase activity is cyclical, peaking in late anaphase/telophase (Fenton and Glover, 1993).

1.5.3.3 Embryonic lethals

The third class of mutants commonly distinguished are those in genes whose zygotic activation is required in embryonic divisions following cycle 13. This is either because the maternal complement becomes depleted at the time of cellularisation, or possibly as a consequence of a novel requirement in cellular mitoses. As these divisions are the first to be zygotically regulated it is predicted that this restricted class of genes will include critical regulators of cell cycle progression.

Embryonic lethal *string* mutants, originally isolated on the basis of a cuticle phenotype (Jürgens et al., 1984), exhibit arrest in G2 phase of cycle 14 (Edgar and O'Farrell, 1989). *string* is the functional homologue of the *S. pombe cdc25* phosphatase (Jimenez et al., 1990) proposed to control entry into mitosis in cycles 14-16 by removing the inhibitory phosphate at tyrosine 15 of $p34^{cdc2}$ (Edgar and O'Farrell, 1990; Edgar et al., 1994) (Section 1.4.1.2).

Mutants in *pebble* are defective in cytokinesis beginning in cycle 14 of embryogenesis. The syncytial divisions, cellularisation, and cycle 14-16 mitoses all proceed (Hime and Saint, 1992), but no contractile rings are apparent (Lehner, 1992), leading to the production of large multinucleate cells.

Embryos mutant for *pimples* have about half the normal complement of cells. Mitosis is defective, commencing in cycle 15, but DNA replication continues producing polyploid nuclei (Smith et al., 1993). In these respects the mutant phenotype of *pimples* is indistinguishable from that of *three rows* (Section 1.6.4), the subject of this study.

Lesions in the *fizzy* gene cause defects in the formation of a variety of late proliferating tissues. Cells in mitosis of cycle 15 in the ventrally derived epidermis, CNS and PNS arrest at metaphase with lethal consequences (Dawson et al., 1993).

1.5.4 Genes identified by sequence conservation/functional complementation, and reverse genetics

Based on the assumption that all eukaryotes share the same central mechanism of cell cycle control, many essential regulatory genes have been isolated in *D. melanogaster* in the absence of a mutant phenotype. Conservation of gene sequence has enabled the cloning of genes, by screening libraries with heterologous probes, or with PCR based approaches. Functional conservation has also been exploited to obtain homologues of yeast genes by complementation of loss of function mutants. In some instances, following gene characterisation and mapping, "reverse genetic" approaches have been applied to deduce the consequences of mutation for the organism.

The homologue of *S. pombe cdc2* (*Dm cdc2*) has been independently cloned by rescue of the yeast mutant (Jimenez et al., 1990), and by PCR using primers from

evolutionary conserved regions (Lehner and O'Farrell, 1990a). Both the *Dm cdc2* mRNA (Jimenez et al., 1990; Lehner and O'Farrell, 1990a) and protein (Foe et al., 1993; Edgar et al., 1994) are present throughout embryogenesis and neither show cell cycle dependent patterns of expression. Loss of function mutants exhibit a classical late larval lethal phenotype and reduction of maternal contribution has revealed a requirement for *Dm cdc2* at the G2/M transition (Stern et al., 1993). *Dm cdc2* is not required for S phase (Smith et al., 1993; Stern et al., 1993). This function may be conferred by a variant form, *Dm cdc2c* (Lehner and O'Farrell, 1990a), shown by reciprocal immunoprecipitation to associate with the G1 specific cyclin E (Knoblich et al., 1994).

A variant homologue of *cdc25*, *twine*, has been isolated by its ability to complement the *S. pombe* mutant (Jimenez et al., 1990). *twine* has a specific function in meiosis. It is expressed in both male and female gonads, and mutants are defective in gametogenesis (Alphey et al., 1992; Courtot et al., 1992). *twine* is also present during, and necessary for, syncytial embryonic mitoses (Alphey et al., 1992).

Genes for cyclins A and B have been cloned using degenerate primers derived from regions of evolutionarily conserved amino acid sequence (Lehner and O'Farrell, 1989; Whitfield et al., 1989; Lehner and O'Farrell, 1990b; Whitfield et al., 1990). Abundant maternally supplied transcripts of both are present throughout the syncytial divisions. Zygotic transcripts replace the maternal complement as it is lost, for cyclin A in cycles 14 and 15, and for cyclin B abruptly from all but the pole cells during cellularisation (Whitfield et al., 1989). Later in development zygotic transcription is correlated with proliferation in the CNS and PNS (Lehner and O'Farrell, 1990b).

In addition to its concentration in the syncytial pole plasm and persistance in pole cells, the cyclin B message also has a perinuclear distribution during syncytial divisions (Whitfield et al., 1989; Lehner and O'Farrell, 1990b). This distribution is microtubule dependent (Raff et al., 1990), and directed by sequences in the transcript's 3' untranslated region (Dalby and Glover, 1992). Discrete 3' untranslated sequence elements are also responsible for the posterior pole accumulation of maternal cyclin B mRNAs and their translational repression in the pole cells until the resumption of mitosis (Dalby and Glover, 1993).

The marked differences in the temporal and spatial patterns of Cyclin A and B proteins are evidence of distinct roles in the regulation of mitotic progression. In syncytial divisions Cyclin A undergoes rapid turnover that is not cell cycle dependent. Levels of Cyclin B, which are initially stable, undergo progressively greater fluctuations in the syncytial blastoderm cycles as more protein is destroyed in each mitosis (Edgar et al., 1994). Following cellularisation Cyclin A and B each exhibit distinct, highly dynamic, cell cycle dependent patterns of accumulation and degradation (Lehner and O'Farrell, 1990b; Whitfield et al., 1990). Cyclin A levels peak in prophase, decline in prometaphase, and are lost by metaphase. By contrast Cyclin B is degraded in anaphase, consistent with a role in anaphase initiation (but see Section 1.2).

Cyclins A and B can also be distinguished by their subcellular locations. Cyclin A, which is cytoplasmic in interphase, associates with the chromosomes in prophase, and when not degraded in syncytial divisions, remains associated with them until telophase (Maldonado-Codina and Glover, 1992). Cyclin B is associated with the spindle and particularly the centrosomes and astral microtubules (Debec and Montmory, 1992; Maldonado-Codina and Glover, 1992), and with microtubules *in vitro* (Kellogg et al., 1991). Cyclin A degradation proceeds in cells mitotically arrested by treatment with microtubule depolymerising agents but cyclin B levels remain high (Whitfield et al., 1990; Edgar et al., 1994), and centrosomally associated (Debec and Montmory, 1992). The association of cyclin B with the spindle and the dependence of cyclin B degradation on the presence of an intact spindle has been proposed as a basis for the mechanism of checkpoint monitoring of spindle integrity (Foe et al., 1993).

The phenotypic consequences of loss of function in cyclins A and B indicate that their levels do not temporally regulate the cellular divisions and that they act synergistically in the same processes. Cyclin A mutants arrest in S phase of cycle 16, having progressed normally through cycles 14 and 15 on presumed trace amounts of protein derived from residual maternal mRNA (Lehner and O'Farrell, 1990b). Cyclin B mutants exhibit slight delays in the timing of cycles 10 to 13 (Edgar et al., 1994) but are not blocked in cellular divisions (Knoblich and Lehner, 1993). Cell cycle progression continues despite depletion of the maternal cyclin B contribution, although there are

temporal delays and spindle abnormalities. The nondescript phenotype of cyclin B mutants may be explained by the isolation of a variant B type cyclin in *D. melanogaster* (C.F. Lehner, pers. comm.). Evidence for the overlapping and synergistic functions of cyclins A and B comes from the earlier arrest, in G2 of cycle 15, observed in double mutants (Knoblich and Lehner, 1993).

Heterologous screening and complementation approaches have also lead to the identification of two putative G1 cyclins. *DmcycE* the *D. melanogaster* homologue of the human G1 specific cyclin E gene (Section 1.4.1.2) was isolated by library screening with a heterologous probe (Richardson et al., 1993). Overexpression of *DmcycE* is capable of rescuing *S. cerevisiae* deficient in all three G1 cyclins. An additional putative *D. melanogaster* G1 cyclin gene *DmcycC* (*CLNDm*) has been isolated independently by two groups based on its ability to rescue *S. cerevisiae* deficient in G1 cyclin function (Lahue et al., 1991; Leopold and O'Farrell, 1991).

PCR based approaches have also been used to clone six novel KRP encoding genes (Stewart et al., 1991) and provide evidence for the existence of as many as 35 KRP genes in the *D. melanogaster* genome (Endow and Hatsumi, 1991). The map positions of some of these loci correspond to the KRP genes *nod*, *ncd* and *KLP61F* with characterised roles in mitosis (Sections 1 5.2 and 1.5.3.2). Others are close to the position of loci known to affect chromosome distribution (Endow, 1993), making them credible candidate genes.

1.5.5 Genes identified by immunodetection of their encoded proteins

Yet another stategy for identifying products necessary for mitosis is to raise antibodies to subcellular fractions enriched in mitotic components. Two initiatives have exploited contrasting approaches.

A library of monoclonal antibodies has been generated against proteins from nuclear fractions of *D. melanogaster* Kc cells and early embryos (Frasch et al., 1986). These antibodies have revealed the varied subcellular locations of their corresponding antigens and their patterns of redistribution during the nuclear cycle. The gene for one of these antigens, Bx63, which preferentially associates with centrosomes has been isolated by immunoscreening an expression library (Whitfield et al., 1988).

Taxol-stabilised MTs have been used as an affinity matrix to isolate a profile of MT associated proteins (MAPs) from embryos, and antibodies have been generated against 24 of them (Kellogg et al., 1989). These proteins localise in various temporal patterns to mitotic spindles, kinetochores, and many to centrosomes. Coding sequences for one of the centrosomal proteins, D-MAP 190 have been isolated, and been shown to be identical to the Bx63 antigen (Kellogg and Alberts, 1992), demonstrating the convergence of these two approaches. D-MAP 190 is part of a centrosomal complex containing another MAP, D-MAP 60 (Kellogg and Alberts, 1992), and γ-tubulin (Raff et al., 1993) (Section 1.1.1). A further 205kD MAP, localised to spindle and cytoplasmic MTs, has been isolated independently (Irminger-Finger et al., 1990).

It is apparent from this survey of genes involved in mitosis that, despite *D. melanogaster* being the best understood multicellular organism in this regard, the function of very few gene products is understood at the molecular or even subcellular level. The information is fragmentary, even when considering those products studied in detail, and almost nothing is known about the interactions between proteins in dependency pathways and multicomponent complexes. However the sheer number of genes identified, together with the experimental tractability of *D. melanogaster*, represent a valuable resource that promise a major contribution to an understanding of the mechanisms and regulation of mitosis in all eukaryotes. The challenge now is to elucidate the precise function of further essential genes. One of these genes, *three rows*, is considered below.

1.6 The *three rows* gene of *D. melanogaster*

1.6.1 Identification

three rows (*thr*) was identified in a screen for ethyl methane sulphate (EMS) induced (zygotic) embryonic lethal mutations on the second chromosome (Nüsslein-Volhard et al., 1984). Embryonic lethal-bearing lines were scored on the basis of their larval cuticle phenotype. "*three rows*" was so called because of the number of denticle bands on the cuticle of each segment, reduced from the usual wild-type complement of six.

The authors made particular reference to the characteristic and unique morphology of the larval cuticle in alleles of *thr*. With the benefit of hindsight this distinctive phenotype is suggestive of a defect in proliferation leading to a reduction in cell number .

1.6.2 Origin of alleles

Of the nine alleles isolated in the original screen (Nüsslein-Volhard et al., 1984) three remain, thr^{IB}, thr^{IL}, and the temperature sensitive thr^{IIV} (Tearle and Nüsslein-Volhard, 1987). Two additional EMS alleles have been generated, thr^{313} and thr^{321} (R. Tearle, University of Adelaide, Australia).

Of particular value to this study has been the P element allele thr^{BH} generated by P-M hybrid dysgenesis (P. Gergen, SUNY, Stonybrook, NY).

1.6.3 Mapping

The *thr* mutation was originally mapped genetically to the position 2-86, and cytologically, based on *Df(2R)PC4*'s failure to complement, to 55A-F (Nüsslein-Volhard et al., 1984). This places *thr* on the right (long) arm of chromosome 2. Subsequent genetic and cytological mapping located *thr* more precisely to the interval 54F-55A between the proximal breakpoints of *Df(2R)Pcl11B* and *Df(2R)PclW5* (D'Andrea et al., 1993) (Figure 1.6). This placed *thr* between the *grainyhead* (*grh*) locus (Bray and Kafatos, 1991) and the gene for protein phosphatase Y (Dombradi et al., 1989).

The localisation of *thr* between these two previously cloned genes was the basis of the strategy to clone the *thr* coding sequences reported in this study. The region containing *thr* was isolated in a cosmid derived chromosomal walk initiated from the *grh* gene (D'Andrea et al., 1993). Although the mapping of cosmid clones and positioning of the *thr* gene within them was the work of R.J. D'Andrea, this was done concomitantly with the isolation and characterisation of coding sequences reported in this thesis. Accordingly it is described, with appropriate attribution, in Chapter 3.

1.6.4 Mutant phenotype

Other than the clue provided by the mutant cuticle in the original screen, the earliest description of the *thr* phenotype (Tearle and Nüsslein-Volhard, 1987) was that affected embryos had fewer and larger cells, resulting from failure of mitosis after cycle 14.

The *thr* mutant phenotype has been characterised in detail (D'Andrea et al., 1993) and is summarised here.

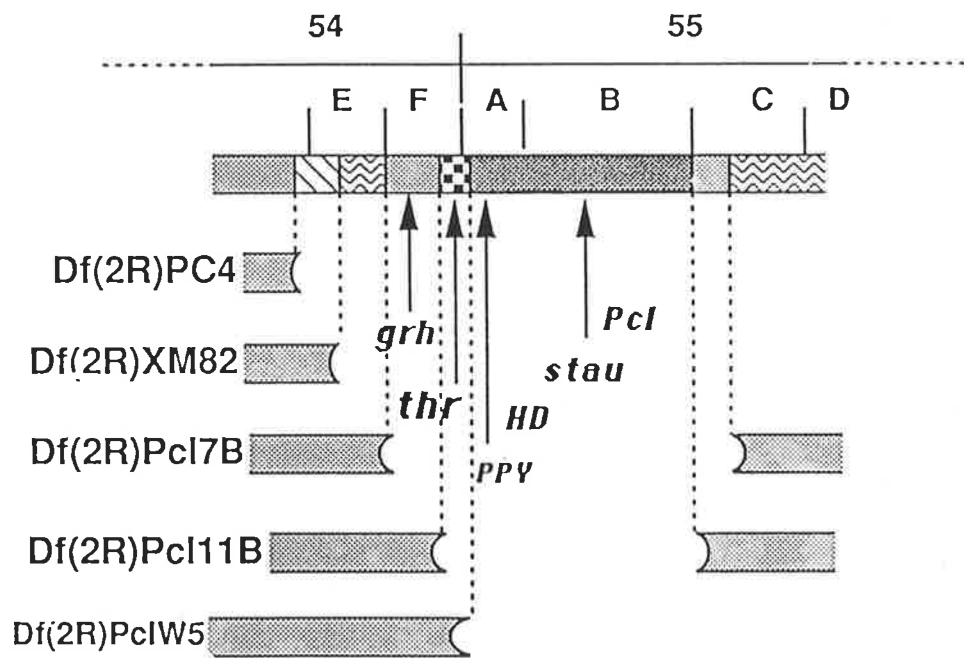
Cyclins A and B have distinct temporal patterns of accumulation and degradation during mitosis that define discrete stages of mitosis (Lehner and O'Farrell, 1990b; Whitfield et al., 1990) (Section 1.5.4). In particular cyclin A is degraded in metaphase before cyclin B. This property has been exploited to characterise the *thr* phenotype by double immunostaining embryos for cyclin A and B (D'Andrea et al., 1993).

An example of an immunofluorescent anti-cyclin A antibody labelling of a wild-type embryo is shown in Figure 1.7A. This embryo was fixed at the stage during which the cells of the dorsolateral epidermis are in the process of mitosis 15. The region of the dorsolateral epidermis outlined with the white box in Figure 1.7A is shown at higher magnification after double labelling with anti-cyclin B antibodies (Figure 1.7C) and a DNA stain (Figure 1.7E). A great number of mitotic figures, including anaphase and telophase figures (see arrowheads and arrows, respectively, in Figure 1.7E) can readily be observed in areas where anti-cyclin B labelling is absent or weak.

An embryo that is homozygous for the *thr* mutant allele thr^{IB} is shown in Figure 1.7B,D,F. Anti-cyclin A antibody labelling (Figure 1.7B) reveal the embryo is at approximately the same developmental stage as the wild-type embryo described above (Figure 1.7A,C,E). A close comparison, however, indicates that the mutant embryo is slightly more advanced in development than the wild-type embryo. The areas in the dorsolateral epidermis that are no longer labelled with anti-cyclin antibodies (Figure 1.7C,D) are somewhat larger in the mutant embryo than in the wild-type embryo. More cells are expected, therefore, to have completed mitosis 15. However, the DNA labelling demonstrates that the completion of mitosis 15 is abnormal in mutant *thr* embryos. In contrast to wild-type, anaphase figures are not found in mutant embryos (Figure 1.7F). Anaphase cells in wild-type embryos can be identified after double

Figure 1.6 Deficiency mapping of *three rows* to 54A-55F (courtesy of R. J. D'Andrea).

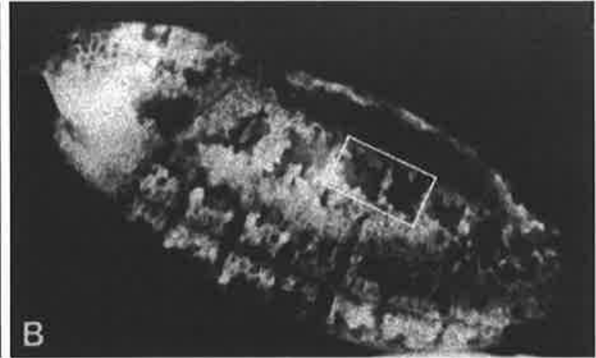
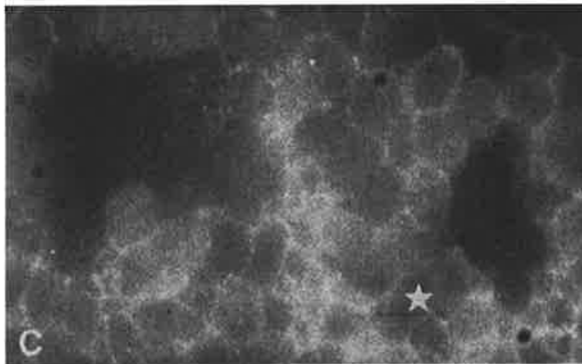
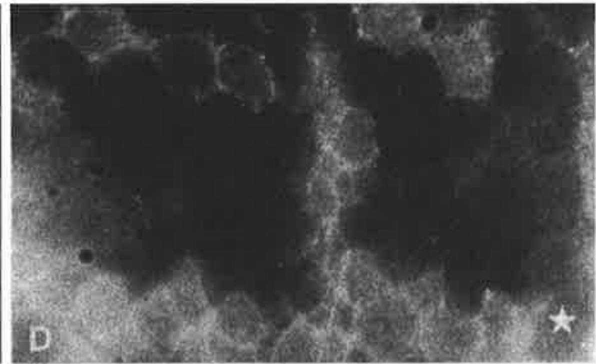
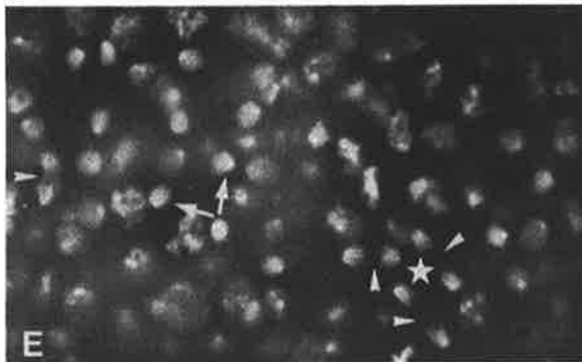
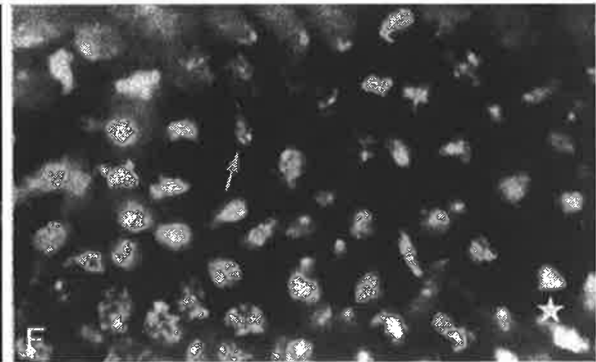
Deficiency breakpoints and the position of selected embryonic lethal complementation groups are shown, with respect to intervals in the 54-55 region. *thr* lies between *grh* and *PPY* loci as defined by the proximal breakpoints of the deficiencies *Df(2R)Pcl11B* and *Df(2R)PclW5* (D'Andrea et al., 1993).



54
55
E
F
A
B
C
D
Df(2R)PC4
Df(2R)XM82
Df(2R)Pcl7B
Df(2R)Pcl11B
Df(2R)PclW5
grh
thr
HD
PPY
stau
Pcl

Figure 1.7 Mitotic defect in *three rows* mutant embryos (from D'Andrea et al., 1993).

Embryos were collected and aged to the stage during which the cells of the dorsolateral epidermis progress through mitosis 15. Progression through mitosis 15 was analysed after double labelling with anti-cyclin A antibodies (A,B), anti-cyclin B antibodies (C,D) and with the DNA stain Hoechst 33258 (E,F). The region outlined in (A) and (B) is shown at higher magnification in (C,E) and (D,F), respectively. In wildtype embryos (A,C,E), anaphase figures (arrowheads) are readily detected in regions where anti-cyclin B labelling is very weak (see star in C,E). In *thr* mutant embryos (B,D,F), anaphase figures are never observed. Anaphase figures are also absent from cells with the weak anti-cyclin B labelling characteristic of anaphase (see star in D,F). During telophase, instead of the pairs of daughter nuclei present in wildtype (see for example arrows in E), a single nucleus is observed in *thr* mutants (see for example arrow in F).

A

B

C

D

E

F

labelling with anti-cyclin antibodies. They are not labelled with anti-cyclin A antibodies, but are still very weakly labelled with anti-cyclin B antibodies (see star in Figure 1.7 C,E). However, in mutant embryos, regions which display the characteristic absence of anti-cyclin A and weak anti-cyclin B labelling of anaphase cells contain no anaphase figures (see star in Figure 1.7 D,F). In addition, normal telophase figures are also absent in mutant embryos. Instead of the characteristic pairs of daughter nuclei with decondensing chromatin, which are observed in wild-type embryos (see arrows in Figure 1.7E), chromatin decondensation is observed only in single nuclei in mutants (see arrow in Figure 1.7F) and not in pairs.

The results of these double labelling experiments indicate that chromosome separation is defective in *thr* mutant embryos (D'Andrea et al., 1993).

Labelling of wild-type and *thr* mutant embryos with anti-tubulin antibodies has confirmed that it is chromosome disjunction that is defective in *thr* mutant embryos (D'Andrea et al., 1993). While anaphase and telophase figuresare readily observed in wild-type embryos *thr* mutant embryos are devoid of normal anaphase and telophase figures. As a consequence of the failure of chromosome disjunction cytokinesis is also defective in mutant embryos.

The analysis of subsequent development in *thr* mutant embryos indicates *thr* function is specifically required for chromosome disjunction and not for cell cycle progression (D'Andrea et al., 1993). Following the failure of chromosome disjunction in cycle 15 other processes, such as chromosome decondensation in cycle 15, and S phase and M phase in cycle 16 are not affected and continue normally.

Detailed examination suggests the mitotic spindle in *thr* mutant embryos is fully functional at least until metaphase (D'Andrea et al., 1993). Spindle formation and chromosome alignment in metaphase of cycle 15 appear normal in *thr* mutant embryos However, only rudimentary signs of spindle elongation are observed during anaphase, in association with failure of chromosome disjunction. Spindle formation also occurs in mitosis 16 but some of these spindles are tripolar or tetrapolar suggesting that, in common with the cell cycle, the centriole cycle is unimpeded in *thr* mutant embryos.

The phenotypic analysis of different *thr* alleles also provides evidence of maternal contribution. There is a difference in the time of onset of phenotypic manifestations in embryos transheterozygous for the amorphic allele thr^{IB} and the partially functional temperature sensitive allele thr^{IIV}, depending on which allele is provided maternally. As the only differences between these two types of progeny are the maternal versus paternal genotype, the difference in phenotype is accounted for by a maternal effect of the *thr* gene (D'Andrea et al., 1993).

1.7 This study

Phenotypic characterisation of the *thr* mutant had revealed a number of distinctive features, particularly the early embryonic lethality and the failure of chromosome disjunction without impediment of cell cycle progression. At the commencement of this study I wished to account for these observations, and elucidate the function of *thr*, at the molecular level. This thesis reports the analysis of the *thr* gene and its product. The results of these investigations are divided into four sections.

The first section ("Prophase") reports on the isolation and characterisation of the *thr* gene. Molecular cloning of *thr* has been unequivocably demonstrated and the sequence of the entire coding region determined. The product encoded is unlike any known to date but includes motifs consistent with function in mitosis. Three rows therefore defines a previously unknown activity essential for mitotic chromosome disjunction.

The second section ("Prometaphase") describes the temporal and spatial patterns of *thr* transcription. These were determined by Northern, RNase protection and primer extension analysis, and by *in situ* hybridisation. They have revealed the pattern of *thr* expression in relation to the cell cycle and with major phases of embryonic and post embryonic proliferation, and provide an account for the cycle 15 mutant defect.

The application of three rows specific antibodies is described in the third section ("Metaphase"). These have detected a temporally and spatially dynamic pattern of *in situ* antigen distribution. Cell cycle dependent localisation of three rows to a specific

component of the mitotic apparatus implicates three rows function in an aspect of chromosome separation or movement.

In the fourth section ("Anaphase") I recount efforts to characterise a diverged *thr* homologue that will reveal domains, conserved over the course of evolution, due to functional constraints on their amino acid sequence. To this end the degree of evolutionary conservation of homologous sequences has been determined and a diverged *thr* homologue has been isolated. Analysis of the homologue suggests that *thr*, a gene essential in *D. melanogaster* for the universal process of mitosis, is not highly conserved.

In the concluding discussion ("Telophase") the contribution of this work to our understanding of mitosis in eukaryotes is considered and experiments are suggested that will further elucidate the function of *thr*.

Chapter 2 (G2/M): Materials and Methods

2.1 Materials

2.1.1 Chemical reagents

Acrylamide:	Bio-Rad
bisacrylamide	
Actinomycin D:	Sigma
Agarose	
Ampicillin	
ATP	
chloramphenicol	
Coomassie blue R-250	
BCIG:	Boehringer Mannheim
BCIP	
digoxigenin-11-dUTP	
dNTPs	
NBT	
Sepharose CL-6B:	Pharmacia
nitrocellulose (BA85)	Schleicher and Schuell

All other reagents were of analytical grade, or the highest grade obtainable.

2.1.2 Enzymes

Restriction endonucleases:	Boehringer Mannheim, New England Biolabs, Pharmacia
Alkaline calf intestinal phosphatase:	Boehringer Mannheim
AMV reverse transcriptase	
Proteinase K	
T4 DNA ligase:	Bresatec
Klenow	
T4 Polynucleotide Kinase	
DNase	Sigma
RNase	

2.1.3 Radio-labelled compounds

α-^{32}P-dATP (3000Ci/mmole):	Bresatec
α-^{35}S-dATP (1500Ci/mmole)	
α-^{32}P-UTP (3000Ci/mmole)	
γ-^{32}P-ATP (4000Ci/mmole)	

2.1.4 *E. coli* strains

LE392: F^-,*hsd*R574, (r_K^-, m_K^+), *sup*E44, *sup*F58, *lac*Y1, *gal*K2, *gal*T22, *met*B1, *trp*R55 (Murray et al., 1977)

DH5α: F^-, ϕ80, *lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, (r_K^-, m_K^+), *sup*E44, *rel*A1, *deo*R,Δ(*lac*ZYA-*arg*F) U169 (Hanahan, 1983)

BL21(DE3): *hsdS*, *gal* (λ*c*I*ts*857, *ind*1, *S*am7, *nin*5, *lac*UV5-T7 gene*1*)

(Studier and Moffatt, 1986)

BL21(DE3) p*lys*S (Studier et al., 1990)

2.1.5 *Drosophila* strains

Unless otherwise indicated, strains are as described (Lindsley and Zimm, 1992) and obtained from the Indiana Stock Centre, Bloomington, IA.

a) *D. melanogaster*

Wild-type strain:

Canton-S

thr alleles:

thr^{IL}, thr^{IB} and thr^{IIV} (Nüsslein-Volhard et al., 1984) (were provided by C. Nüsslein-Volhard, MPI, Tübingen, Germany)

thr^{BH} (P. Gergen, SUNY, Stonybrook, NY)

thr^{313}, thr^{321} (R. Tearle, University of Adelaide, Australia)

All were crossed to the isochromosomal stock *cn bw sp* (R. Tearle)

Balancer chromosomes:

CyO

CyO *wg* P[*lacZ*] (N. Patel, Carnegie Institute of Washington, Baltimore, MD)

SM6a

Deficiency chromosomes:

Df(2L)PC4 (G. Jürgens, MPI, Tübingen, Germany)

Df(2L)Pcl7B (I. Duncan, Washington University, St Louis, MO)

Strains employed in transformation:

w^{1118}

w^{1118}; *cn* thr^{IB} *bw/SM6a* (R. Tearle)

b) *Drosophila* species

D. simulans (National *Drosophila* Species Resource Centre, Bowling Green, OH)

D. yakuba

D. erecta

D. eugracilis

D. ananassae

D. virilis

D. robusta

D. hydei

D. funebris

2.1.6 Media and buffers

a) Media

All buffers and media were prepared with distilled and deionised water and sterilised by autoclaving, except heat labile reagents, which were filter sterilised.

All bacterial strains were propagated in L-broth or on L-agar plates, except LE392 which was grown in T-broth or on T-broth plates.

L-broth: 1% (w/v) amine A
0.5% yeast extract
1% NaCl, pH7.0

T-broth: 0.5% NaCl
1% tryptone

Plates: L or T-broth with 1.5% bacto-agar.

Where required for selection ampicillin was added to a final concentration of 100 μg/ml, and chloramphenicol to 30 μg/ml.

All *Drosophila* strains were grown on *Drosophila* culture media:

10% treacle
20% yeast
1% agar
10% polenta
2.5% tegosept
1.5% propionic acid

b) Buffers

Commonly used buffers were:

Blocking mix:
5% Blotto
1 x PBS
0.1% Tween 20
0.02% sodium azide

Protein gel running buffer :
1.5% tris base
7.2% glycine
0.5% SDS

Protein gel load buffer:
62.5 mM Tris-HCl pH 6.8
10% glycerol
2% SDS
5% 2-β-mercaptoethanol
0.00125% bromophenol blue

PBS:
7.5 mM Na_2HPO_4
2.5 mM NaH_2PO_4
145 mM NaCl

PSB:
10 mM Tris-HCl pH 7.4
10 mM NaCl
100 mM $MgCl_2$

TBE:
50 mM Tris-borate pH 8.3
1 mM EDTA

TBS: 20 mM Tris-HCl pH 7.5
0.5 M NaCl

TE: 10 mM Tris-HCl pH 7.4
0.1 mM EDTA

TAE: 40 mM Tris-acetate pH 8.2
1 mM EDTA

10 x agarose gel load buffer: 50% sucrose
50 mM EDTA
0.1% bromophenol blue

Sequencing gel load buffer: 98% deionised formamide
10 mM EDTA pH 8.0
0.025% xylene cyanol
0.025% bromophenol blue

SSC: 150 mM NaCl
15 mM Na citrate

2.1.7 Libraries

pNB40cDV 0-4, 4-8 h embryonic cDNA libraries (Brown and Kafatos, 1988)
λ gt11 0-18 h embryonic cDNA library (Clontech)
λEMBL3 *D. erecta* genomic library (Hickey et al., 1991)

2.1.8 Plasmids

a) Cloning vectors

pCaSpeR4 (V. Pirotta, University of Geneva, Switzerland)

pBluescript (Stratagene)

pGEX-2T (Smith and Johnson, 1988)

pRK171 (A.H. Rosenberg, Brookhaven, NY)

b) Recombinant plasmids

pπ25.7*wc*(Δ2-3) (G. Rubin, UC Berkeley, CA)

2.1.9 Oligonucleotides

a) Sequencing Primers

M13 -20: 5'-d(GTAAAACGACGGCCAGT)-3'

Reverse: 5'-d(AACAGCTATGACCATG)-3'

T3: 5'-d(ATTAACCCTCACTAAAGGGA)-3'

T7: 5'-d(TAATACGACTCACTATAGGG)-3'

SP6: 5'-d(GAATTTAGGTGACACTATAG)-3'

b) Oligonucleotide for primer extension

2913: 5'-d(GCTTCTGCAGCAGCACATGG)-3'

(complementary from 475 to 494, Figure 3.4)

2.1.10 Molecular weight markers

a) DNA

λ DNA digested with *Bst*EII and *Sal*I produces fragments of sizes (in kb): 14.14, 7.24, 4.82, 4.32, 3.68, 3.13, 2.74, 2.32, 1.93, 1.37, 1.26, 0.70, 0.45, 0.22 and 0.11

b) RNA

0.24-9.5 kb RNA Ladder (GIBCO BRL)

c) Protein

Prestained high molecular weight markers (GIBCO BRL)

2.2 Methods

2.2.1 λ bacteriophage propagation

LE392 plating cells were prepared by resuspending a mid-log phase culture grown in T-broth, 10 mM $MgCl_2$, in 0.5 volume 10 mM $MgCl_2$. Appropriate dilutions of λ phage were added to 100 μl LE392 plating cells, mixed with 3 ml molten 0.7% agar at 42°C, poured onto 85 mm T-broth plates and incubated at 37°C for 8-16 h. For 140 mm plates volumes were scaled up three fold.

Agar plugs containing plaques of interest were removed with narrow or wide end of Pasteur pipette and phage eluted in 1 ml PSB at rt for 4 h. Serial dilutions for plating were made in PSB. Phage stocks were stored at 4°C with addition of 50 μl $CHCl_3$.

2.2.2 λ bacteriophage library screening

Phage DNA on 140 mm T-broth plates at a density of 5 x 10^4 pfu/plate was transferred to duplicate sets of "Plaquescreen" (New England Nuclear) filters by overlaying. Orientation marks were made and filters where processed according to manufacturer's instructions. Filters were hybridised in plastic petri dish using procedures in 2.2.6.

2.2.3 Isolation of λ bacteriophage DNA

Plugs of single plaques were transferred into 0.5 ml of stationery phase LE392 culture + 16 µl $MgSO_4$ and incubated at 37°C for 30 min. This was transferred to 10 ml T-broth and incubated at 37°C with shaking until lysis (usually 4 h) whereupon 0.5 ml $CHCl_3$ was added for a further 10 min. DNase and RNase to 10 µg/ml were added for 30 min at 37°C and debris pelleted by spinning for 5 min at 1500g. 0.5 g PEG 6000 and 0.44 g NaCl were gently dissolved in the supernatant and left at 4°C for 16 h.

Phage were pelleted at 10,000 g, 4°C, for 10 min and resuspended in 500 µl PSB, 20 mM EDTA, 50 µg/ml proteinase K, 0.5% SDS at 65°C for 1 h. λ DNA was extracted with phenol/$CHCl_3$ twice and ethanol precipitated at rt. High molecular weight DNA was "spooled" off and resuspended in 100 µl TE. Typical yield was 10 µg.

2.2.4 Plasmid library screening

DH5α cells transformed (Section 2.2.12) with plasmid library DNA were plated at a density of 5 x 10^4 colonies/plate onto nitrocellulose filters overlayed on 140 mm L-broth plates, and grown at 30°C for 16 h. Replica filters were made and processed as described (Brown and Kafatos, 1988). Filters were hybridised in plastic petri dishes using procedures in section 2.2.6.

Master filters were stored on L-broth plates containing ampicillin supplemented with 10% glycerol, at -20°C.

2.2.5 Radiolabelling of DNA fragments

DNA fragments were labelled by Klenow catalysed α-^{32}P-dATP incorporation in random oligonucleotide primed synthesis products (Feinberg and Vogelstein, 1983) using a "Megaprime" kit (Amersham). Unincorporated nucleotides were removed by size exclusion chromatography on Sepharose CL-6B mini column (Section 2.2.13).

2.2.6 Hybridisation of radiolabelled probes to membrane immobilised nucleic acids

Filters were pre-hybridised with hybridisation mix (50% formamide, 5 x SSC, 0.5% blotto, 100 µg/ml sonicated and denatured salmon sperm DNA) at 42°C for at least 2 h. If nylon based filters (Plaquescreen) were used hybridisation mix was supplemented with 1% SDS. Heat denatured and snap cooled radiolabelled probe was added to the membranes with fresh hybridisation mix and incubated at 42° for 4-16 h.

Membranes were washed typically (at high stringency) with two 10 min washes each of 2 x SSC, 0.1% SDS at rt, then 0.1 x SSC, 0.1% SDS at 65°C.

2.2.7 Autoradiography

Membranes or dried gels were exposed for variable periods to X-Omat AR X-ray film (Kodak) in an autoradiography cassette (Ilford) at rt, or at -80°C in the presence of a calcium tungstate intensifying screen.

2.2.8 "Miniprep" isolation of plasmid DNA

A single colony was used to innoculate 10 ml of L-broth plus ampicillin and incubated for 5-16 h at 37°C with shaking. DNA was isolated by the "boiled lysis" method (Murphy and Kavanagh, 1988) to the stage of isopropanol precipitation, where the pellet (typical yield 50 µg) was resuspended in 50 µl of TE. Proportional scaling down of volumes was used in minipreps of smaller cultures.

2.2.9 Restriction analysis of DNA

DNA was digested with restriction endonucleases under conditions recommended by the suppliers. 1/10th volume of agarose gel load buffer was added and samples were run on a 1.2% agarose horizontal minigel (Hoefer HE 33) in TAE buffer at 5-10 V/cm. DNA was visualised by staining the gel with 10 μg/ml ethidium bromide and viewing under UV light.

2.2.10 DNA fragment purification

DNA was isolated from agarose gel slices by centrifugation at 12,000g through 25 μl of acid washed glass beads in a 0.5ml microfuge tube and collected in a 1.5 ml microfuge tube. Contaminating micromolecules were removed on a Sepharose CL-6B mini column (Section 2.2.13).

2.2.11 Creation of recombinant plasmids

Plasmid vector DNA was prepared by digestion with the appropriate restriction endonuclease in the presence of 1 U CIP to remove 5' terminal phosphates. Linear vector molecules were then purified on Sepharose CL-6B mini-columns (Section 2.2.13). Ligations of 100 ng total DNA were performed with insert:vector of 3:1 in 10 μl 0.05 M Tris-HCl pH 7.5, 0.01 M $MgCl_2$, 0.01 M DTT, 1 mM ATP and 1 U T4 DNA ligase at 14°C for 4-16 h.

2.2.12 Transformation of recombinant molecules

A 50 ml mid-log phase culture of DH5α (or BL21(DE3)) was harvested, resuspended in 20 ml 50 mM $CaCl_2$ and left on ice for 20 min. The cells were harvested and carefully resuspended in 2 ml 50 mM $CaCl_2$. 100 μl cell suspension was typically mixed with 1 μl ligation mix and left on ice for 30 min before heat shock at 37°C for 3 min. The mixture was incubated at 37°C for 30 min following addition of 0.5 ml L-broth, plated on L-broth plates with ampicillin (and with chloramphenicol for BL21(DE3)p*lys*S), and grown at 37°C for 16 h. For "blue/white" selection of pBluescript recombinant clones 10 μl each of 20% IPTG and 10% BCIG was added to plating mixture.

2.2.13 Nucleotide sequence analysis

a) Generation of nested deletions

Recombinant pBluescript plasmid DNA was restriction digested at combinations of unique sites in their polylinker sequences to generate linear molecules with 5' overhangs adjacent to the insert and 3' (protecting) overhangs at the opposite ends. Additional Sepharose CL-6B purifications (Section 2.2.13) were done prior to, and following restriction digestion. Time courses of Exonuclease III digestion were performed using a Double Stranded Nested Deletion Kit (Pharmacia). Digestion was assayed by agarose gel electrophoresis and suitable timepoints where chosen for ligation and transformation (Sections 2.2.11 and 12). Colonies were "miniprepped" (Section 2.2.8) for insert size analysis and sequencing.

b) Sequencing template preparation

9 µg of plasmid DNA was RNase treated, alkali denatured and purified on a Sepharose CL-6B mini column as described (Murphy and Kavanagh, 1988). 3 µg of this was annealed with 10 ng of primer at 37°C for 1 h.

c) Sequencing reactions

DNA was sequenced by the dideoxy method (Sanger et al., 1977), using α-^{35}S-dATP and a Sequenase Sequencing Kit (United States Biochemical).

d) Electrophoresis

Products of sequencing reactions were resolved on 0.4 mm 6% acrylamide (acrylamide:bis-acrylamide, 20:1), 8 M urea, TBE gels. Gels were fixed in 10% acetic acid, 20% methanol, transferred to 3MM paper (Whatmann), dried on a vacuum gel drier at 80°C for 30 min, and autoradiographed for 16 h.

e) Sequence Analysis

Sequence analysis and comparisons were carried out using computer programs of Staden (1980) and the Genetics Computer Group (University of Wisconsin, WI). Searches of GenBank and EMBL databases used the FASTA (Pearson and Lipman, 1988) and BLAST (Altschul et al., 1990) programs.

2.2.14 Maintainence of *Drosophila* stocks

Stocks were routinely cultured at 25°C in plastic vials or half-pint glass bottles containing *Drosophila* culture medium. Stocks needed for collections of large numbers of eggs were maintained in a population cage.

2.2.15 Genetic transformation

The 11.0kb *Not*1 fragment shown in Figure 3.1 was subcloned into the w^+ transformation vector pCaSpeR4. DNA was purified using QIAGEN-tip 100 columns (QIAGEN Inc.). Transformation construct was co-microinjected with pπ25.7*wc*(Δ2-3) (as a source of transposase activity) at concentrations of approximately 1 μg/ml into the posterior end of manually dechorionated 45-90 min AED w^{1118} embryos.

Adults that developed from the injected embryos were crossed to w^{1118}; *cn* thr^{IB}*bw/SM6a* and transformed lines recovered as indicated by w^+ eye colour. In all, three transformed lines were crossed to another *thr* mutant line to generate individuals carrying the transformed fragment in a *cn* thr^{313} *bw/cn* thr^{IB} *bw* background.

2.2.16 Egg collects

Eggs were collected and aged on apple juice agar plates at 25°C before removal of the chorion with 4% hypochlorite. Embryos were snap frozen and stored at -80°C for RNA extraction, or fixed as below.

2.2.17 Fixation of embryos for *in situ* hybridisation and immunostaining

Embryos were fixed and devitellinised as described (Karr and Alberts, 1986) and stored at -20°C in methanol, 5% EGTA pH 7.5 (Kellogg et al., 1988).

2.2.18 Expression studies

a) RNA isolation

RNA was extracted from staged embryos by homogenisation in 6 M guanidine-HCl, 0.1 M CH_3COONa, and pelleting at 37,000 rpm in a SW41 rotor for 16 h through a 4.8 M CsCl pad (MacDonald et al., 1987). Pellets were resuspended and ethanol precipitated twice before being resuspended in H_2O and yield quantified by reading absorbance at 260 nm.

b) Northern analysis

20 µg of total RNA from each life cycle stage was fractionated on low formaldehyde, 1.2% agarose gels (Ausubel et al., 1987). RNA was immobilised by blotting onto Nytran-N (Schleicher and Schuell) with HETS buffer (Cinna/Biotecx). Evenness of loading and transfer efficiency was assayed by staining membrane with methylene blue (Herin and Schmidt, 1988). Filters were hybridised with radiolabelled probe, washed and autoradiographed (Section 2.2.6.7).

c) RNA probe synthesis

An antisense RNA probe was synthesised by T7 RNA polymerase incorporation of α-^{32}P-UTP from the template of a *Sal*I digested 421 bp genomic *Hin*dIII fragment (nucleotides -326 to 96, Figure 3.4) subcloned into pBluescript, using a Message Maker *in vitro* Transcription Kit (Bresatec), according to instructions.

Reaction was resolved by electrophoresis on a 14 x 14 x 0.5 cm 6% acrylamide (acrylamide:bis-acrylamide, 20:1), 8 M urea, TBE gel. The primary product of the labelling reaction was detected by autoradiography, the corresponding region of the gel excised and the RNA probe eluted into 500 µl 500 mM CH_3COONH_4, 1 mM EDTA, 0.1% SDS, 10 U RNase Inhibitor for 16 h at 37°C.

d) RNase protection analysis

A Ribonuclease Protection Assay Kit (Ambion) was used according to instructions. 5 μg of total RNA from each stage (with yeast RNA as a control) was co-ethanol precipitated with 50,000 cpm of RNA probe. The mixture was resuspended, allowed to hybridise, RNase treated, phenol/$CHCl_3$ extracted and ethanol precipitated. Reaction products were resolved on a sequencing gel (Section 2.2.13) with sequencing reactions as approximate size standards.

e) Radiolabelling of oligonucleotide

Oligonucleotide 2913 was labelled in a 10μl reaction containing 500 ng oligonucleotide, 6 μl γ-^{32}P-ATP, 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1mM DTT, 0.1 mM spermidine, 0.1 mM EDTA and 3 U T4 Polynucleotide Kinase for 1 h at 37°C.

Products of labelling reaction were resolved on a 14 x 14 x 0.5 cm 20% acrylamide (acrylamide:bis-acrylamide, 20:1) TBE gel. The primary product of labelling reaction was detected by autoradiography, the corresponding region of the gel excised, and the oligonucleotide eluted into 500 μl TE for 1 h at 65°C.

f) Primer extension analysis

1 μl labelled oligonucleotide was co-ethanol precipitated with 20 μg of total RNA from each stage and resuspended in 10μl 10 mM Tris-HCl pH 8.3, 200 mM NaCl. The mixture was denatured at 80°C for 3 min and slowly cooled to 42°C. The mixture was made to 10 mM Tris-HCl pH 8.3, 60 mM NaCl, 10 mM $MgCl_2$, 500 μM dNTPs, 50μg/ml Actinomycin D, 24 U AMV reverse transcriptase in 35μl total volume, and incubated at 42°C for 1 h. 0.5 μg of RNase A was added for 15 min at 37°C, the mixture extracted with phenol:$CHCl_3$ (1:1), and with $CHCl_3$, and ethanol precipitated. Products were resolved by electrophoresis (Section 2.2.13) with sequencing reactions as size markers.

g) Whole mount *in situ* hybridisation to mRNA

200 ng of*AluI*/*HhaI*/*HaeIII*/*RsaI* digested pUJA8 cDNA clone was labelled by random priming with 10 times the normal concentration of oligonucleotides (C. Oh and

B. Edgar; unpublished modifications of Feinberg and Vogelstein, 1983) and incorporation of digoxigenin-11-dUTP. Whole-mount *in situ* hybridisations were performed with staged Canton-S and *Df(2L)PC4* embryos according to modifications (Lehner and O'Farrell, 1990b) of the protocol (Tautz and Pfeifle, 1989). Embryos were mounted for microscopy in 80% glycerol.

2.2.19 Electrophoresis of proteins

Proteins were resolved on discontinuous SDS-polyacrylamide gels (Laemmli, 1970) in a Mini-PROTEAN II cell (Bio-Rad) according to instructions. Samples were diluted at least 1:4 with protein gel load buffer and heated at 100°C for 3-5 min before resolution on 7.5% acrylamide (acrylamide:bis-acrylamide, 37.5:1) gels at 200 V for 45 min.

For analytical purposes typical 10 µl sample volumes per track were run on 0.75 mm thick gels and visualised by staining in 0.1% Coomassie blue, 40% methanol, 10% acetic acid for 30 min and destaining in 40% methanol, 10% acetic acid. For preparative purposes 500 µl sample volumes were run across 1.5 mm thick gels and visualised by staining in 0.1% Coomassie blue/H_2O for 5 min and destaining in H_2O.

2.2.20 Bacterial expression of *thr* derived protein

a) T7 system

The 2.0 kb *NdeI*/*Bst*YI fragment of UJA8 (nucleotides 1914-3923 in Figure 3.4) was cloned (Section 2.2.11) into *NdeI*/*Bam*HI digested pRK171, and used to transform BL21(DE3) p*lys*S (Section 2.2.12).

To induce expression a single colony was inoculated directly into 500 ml L-broth with ampicillin and chloramphenicol and grown with shaking at 37°C till mid-log phase (usually 4 h). IPTG to 0.84 mM was added and incubation continued for a further 2 h. Cells were harvested, resuspended in 5 ml PBT and sonicated on ice 10 times for 30 sec every min. The lysate was spun at 12,000g in a microfuge and both pellet and supernatant retained.

b) Glutathione S-transferase fusion protein

The 3.5 kb *Bgl*II/*Not*I fragment of UJA8 (from nucleotide 1149 in Figure 3.4), including 14 nt of pNB40cDV, was cloned (Section 2.2.11) into *Bam*HI/*Sma*I digested pGEX-2T (Smith and Johnson, 1988). UJA8 was first cut with *Not*I, the 5' overhang filled in with 0.5 mM dNTPs, 1 U of Klenow at 37°C for 30 min, then cut with *Bgl*II, and the fragment isolated. The clone was transformed into BL21(DE3) (Section 2.2.12).

Expression of the fusion protein was induced as described (Smith and Johnson, 1988) and as for T7 protein (above).

2.2.21 Antibody production

T7 and GST fusion protein preps were run on two preparative gels each, to the limit of the gels resolving power caused by overloading. Following staining the gel slices were excised, an equal volume (usually 1.5 ml) of PBS was added and the gel slices were homogenised with a tissue homogeniser. The homogenate was mixed with an equal volume of span 85:paraffin oil (1:5) and emulsified by passing between syringes through 20G hypodermic needles.

Three New Zealand white rabbits were pre-bled, inoculated at 4 subcutaneous points with 2 ml total (containing about 1 mg of purified protein) of the emulsion, and boosted 3 times at monthly intervals with similar quantities. 10-14 days after the final boost rabbits were terminally bled.

2.2.22 IgG purification

The IgG fraction from rabbit preimmune sera was purified using an Affi-Gel Protein A MAPS II kit (Bio-Rad) according to instructions except that the bound IgGs were eluted and neutralised as for high affinity elution in 2.2.24.

2.2.23 Construction of affinity column

T7 protein preps were run on four preparative gels. The lateral margins of each gel were excised and stained to locate the T7 expressed protein, and the corresponding regions were isolated. Protein was electroeluted from gel slices into 1:10 protein gel

running buffer in dialysis tubing. Dialysis tubing was placed in a horizontal gel tank of protein gel running buffer and run at 12 mA and 3 W for 16 h. Buffer replacement with 50 mM HEPES, 25 mM KCl, 0.1% Tween 20 pH 7.6 was done by dialysis with 4 changes of 1 l over 36 h.

Yield was quantified by measuring absorbance at 280 nm relative to BSA solutions of known concentration.

The purified protein (about 6 mg) was coupled to 1 ml of Affigel 10 agarose (Bio-Rad) according to the manufacturer's instructions. Efficiency of coupling was assayed on Coomassie stained protein gels. A column was poured using a 5 ml plastic syringe with a glass microfibre filter (Whatman), cut to size, at the base. Prior to initial use the column was subjected to treatment by all the solutions used in 2.2.24, to strip any unbound antigen. The column was stored at -20°C in PBS containing 0.02% sodium azide, 50% glycerol.

2.2.24 Affinity purification of antibodies

Purification was performed at rt by gravity flow as described (Kellogg and Alberts, 1992). 5 ml of serum was centrifuged at 100,000 g to remove any particulate matter and applied repeatedly to the column over 2 h. The column was washed with 50 ml TBS to remove nonspecifically bound protein. Low affinity antibodies were first eluted by the application of 1.4 M $MgCl_2$, 10% glycerol, 50mM HEPES. High affinity antibodies were eluted with 0.5% acetic acid, 0.15 M NaCl by sequential application of 0.5 ml aliquots, collection in microfuge tubes, followed by immediate neutralisation with 5-50 µl 1 M Na_2PO_4, and addition of 5 µl 2% sodium azide. The column was finally stripped with 100 mM triethylamine pH 11.5 and neutralised with TBS. Column fractions were assayed for the presence of antibody activity by western analysis on bacterially expressed protein (Section 2.2.25). Peak fractions were pooled and concentrated by dialysis into PBS containing 0.02% sodium azide, 50% glycerol.

2.2.25 Western analysis

a) Sample preparation

1-5 ug of bacterially expressed protein was run on 0.75 mm preparative gels. For analysis of *Drosophila* embryonic protein approximately 20 methanol fixed (Kellogg et al., 1988) embryos in 10 µl of protein gel load buffer were loaded per gel track.

b) Blotting

Following electrophoresis gels were equilibrated for 1 h in two changes of 100 ml transfer buffer (48 mM Tris base, 39 mM Glycine, 0.0375% SDS, 20% Methanol) and electrophoretically transferred to nitrocellulose at 25 V, 3 mA/cm^2 of gel, for 1 h using a Trans-Blot SD Transfer Cell (Bio-Rad) according to instructions. After transfer, orientation marks were made on the nitrocellulose and it was allowed to air dry and, where appropriate, cut into strips.

c) Immunodetection

All steps were performed at rt with mixing by oscillation. Blots of bacterially expressed protein were rinsed twice in PBS, incubated in blocking mix for 1 h, then in 1:200-500 dilution of antibody in 100-200 µl blocking mix/cm^2 for 2 h. Nitrocellulose was washed four times with PBS for 5 min and incubated with 1:500 dilution of biotinylated anti-rabbit Ig antibody (Amersham) in blocking mix for 2 h. Following washing the membrane was incubated with a 1:5000 dilution of streptavidin-AP conjugate (Boehringer Mannheim) for 30 min, and washed again. The blot was equilibrated in two rinses of 100 mM Tris-HCl pH 9.5, 1 mM $MgCl_2$ and chromogenic reaction performed in the dark with 50 µl/cm^2 of 0.3 mg/ml BCIP, 0.3 mg/ml NBT, 100 mM Tris-HCl pH 9.5, 1 mM $MgCl_2$. Reaction was stopped by rinsing in 20 mM EDTA.

Immunodetection of embryonic protein blots was performed identically except washes were with PBT for 20 min each, tertiary compound was from Peroxidase standard ABC Kit (Vectastain) and detection was with ECL Gene Detection reagents (Amersham), each according to instructions.

2.2.26 Immunostaining of embryo whole mounts

Fixed embryos were progressively rehydrated through to PBT, left in PBT for 1 h, and in blocking mix for 2 h. Embryos were incubated in 10 x their volume of 1:50 dilution of affinity purified antibody in blocking mix at 4°C for 16 h, with rocking to maintain suspension. All subsequent steps were performed at rt. Washes after each incubation consisted of four quick changes of PBT followed by two washes of 1 h with rocking. The secondary antibody was first preabsorbed against an identical batch of untreated embryos at the final concentration (1:100) before incubation for 4 h.

Following incubation with Texas Red conjugated sheep anti-rabbit IgG (Jackson), as a secondary antibody, embryos were counterstained for DNA with chromomycin A_3 (Sigma) as described (Foe, 1989).

With biotinylated sheep anti-rabbit Ig (Amersham), streptavidin-HRP (Vectastain ABC Kit) was applied as the tertiary compound, and HRP activity was detected by incubation with 0.5 mg/ml DAB, 0.045% H_2O_2 and 0.064% $NiCl_2$. DNA was counterstained with 10 μg/ml Hoechst 33258.

Embryos were mounted for microscopy in 80% glycerol.

2.2.27 Image capture

Fluorescent and brightfield microscopy was performed on a Zeiss Axiophot microscope equipped for Normarski and epifluorescence. Objectives used were Plan-Neofluar 20x/0.5, 40x/0.75 and 100x/1.3 oil immersion. Photographs were taken with a Zeiss Microphot system and recorded on Ektachrome 160T film (Kodak). Slides were scanned with a Kodak RFS 2035 Film Scanner.

Confocal microscopy was performed with a Bio-Rad MRC 1000 Confocal Imaging System in conjunction with a Nikon Optiphot microscope. Objectives used were Plan-Apochromat 40x/0.95, and 60x/1.4 and 100x/1.4 oil immersion.

The Adobe Photoshop program was used for image preparation. Colour prints were obtained using a Kodak XLT 7720 Digital Continuous Tone Printer.

2.2.28 Isolation of genomic DNA from adult *Drosophila*

20 adult flies were collected in a microfuge tube and placed on ice. They were macerated with glass pestle in 100 µl of ice cold 0.1 M Tris-HCl pH 9, 0 0.1 M EDTA followed by addition of 100 µl of 2% SDS and incubation at 65°C for 30 min. 42 µl of 5 M CH_3COOK was added, the mixture left on ice for 30 min and cell debris pelleted at 12,000g in a microfuge. The spin was repeated on the supernatant after another 10 min on ice. DNA was pelleted by addition of 120 µl isopropanol and spinning at 12,000g for 10 min in a microfuge. DNA (typical yield 20 µg) was resuspended in 20 µl H_2O.

Routinely, 10 µg was restriction digested and run in each track of a 15 x 13.5 x 0.5 cm horizontal 1.2% agarose gel for Southern analysis.

2.2.29 Southern blotting

After visualisation with ethidium bromide, agarose gels were soaked in 0.25 M HCl, then in 0.5 M NaOH, 1.5 M NaCl until bromophenol blue in the gel turned yellow, then blue respectively. Following neutralisation in 1.0 M Tris-HCl pH 7.4, 1.5 M NaCl for 30 mins the gel was placed in a sandwich that consisted of (from bottom): 5 sheets of 3MM paper drawing on a reservoir of 20 x SSC, the gel, a sheet of nitrocellulose (prewetted in H_2O), 3 sheets of 3MM paper, 5 cm of paper towels, and a glass plate with a 0.5 kg weight upon it. Sandwich was left for 16 hours and then dismantled, with orientation marks made on the nitrocellulose. The membrane was allowed to air dry then baked *in vacuo* at 80°C for 2 h. Radiolabelled probe was hybridised with nitrocellulose as in 2.2.6.

2.2.30 Southern hybridisation under nonstringent conditions

"Genus blot" was hybridised as in 2.2.6 except hybridisation was carried out at 37°C with a mix containing 29 % formamide as suggested (O'Neil and Belote, 1992). Filters were washed in 6 x SSC, 0.1 % SDS at rt.

2.2.31 Regulatory considerations

All manipulations involving recombinant DNA were carried out in accordance with the regulations and approval of the Genetic Manipulation Advisory Commitee and the University Council of the University of Adelaide.

All manipulations involving animals were carried out in accordance with the regulations and approval of the Animal Ethics Committee and the University Council of the University of Adelaide.

2.3 Abbreviations

Abbreviations are as described in "Instructions to authors", *Biochem. J.* (1978) **169**, 1-27.

In addition:

aa	amino acid residues
AP	alkaline phosphatase
APS	ammonium persulphate
BCIG	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bisacrylamide	N,N'-methylene-bisacrylamide
bp	base pairs
BSA	bovine serum albumin
blotto	skim milk powder
CIP	alkaline calf intestinal phosphatase
Da	Dalton
DTT	dithiothreitol
HEPES	N-2-Hydroxyethyl piperazine-N-2-ethane sulphonic acid
HRP	horse radish peroxidase
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
Klenow	(fragment) of *E. coli* DNA polymerase I

Mb	megabase
my	million years
mya	million years ago
NBT	4-nitro blue tetrazolium chloride
nt	nucleotide
ORF	open reading frame
PEG	polyethylene glycol
pfu	plaque forming unit
rpm	revolutions per minute
rt	room temperature
SDS	lauryl sulphate sodium salt (sodium dodecyl sulphate)
TEMED	N,N,N',N'-tetramethylethylenediamine
U	unit

Chapter 3 (Prophase): Gene isolation and characterisation

3.1 Background

As described in the introduction, mutation of the *thr* locus in *D. melanogaster* has been shown to result in the disruption of the embryonic cell cycles (D'Andrea et al., 1993). Specifically, chromosome disjunction and, consequently, cytokinesis are affected in the mutant embryonic mitoses. In embryos homozygous for the strongest *thr* alleles, these mitotic defects first appear during the 15th mitosis after fertilisation. During this mitosis, however, spindle formation, cyclin degradation and DNA decondensation occurs. In addition, a cycle 16 S phase and an abnormal cycle 16 mitosis are observed. Thus cell cycle events appear not to be affected, leading to the conclusion that the primary consequence of a loss of *thr* function is a failure of chromosome disjunction. This phenotype also shows that cell cycle progression in *D. melanogaster* can proceed independent of chromosome disjunction.

Manifestation of the zygotic *thr* mutant phenotype as early as cycle 15 is unusual for a *D. melanogaster* cell cycle gene. Of the large number of genes that are required to complete a cell cycle, zygotic expression of only the *string*, *pimples*, *pebble*, cyclin A, *fizzy* and *thr* genes (Sections 1.4.3.3 and 1.6.4) is known to be required prior to completion of the first 16 cycles, divisions that generate nearly all the larval tissues.

The highly distinctive *thr* mutant phenotype made it a priority to isolate and characterise the gene and its encoded protein, with the aim of understanding its precise function in chromosome disjunction. As described in section 1.6.3, deficiency mapping (D'Andrea et al., 1993) had placed *thr* between the *grainyhead* (*grh*) locus (Bray and Kafatos, 1991) and the gene for protein phosphatase Y (Dombradi et al., 1989).

The genomic region containing *thr* was isolated as a cosmid clone cos3.3a in a chromosome walk initiated from the 3' end of the *grh* gene (D'Andrea et al., 1993). Comparison of the signal intensity when terminal restriction fragments of this cosmid were used to probe genomic DNA isolated from flies heterozygous for *Df(2R)Pcl11B*, relative to

flies carrying an isochromosomal 2nd chromosome, demonstrated that the proximal breakpoint of *Df(2R)Pcl11B* lay within this cosmid (D'Andrea et al., 1993) (Figure 3.1).

In addition, Southern analysis with the terminal 6.5kb *Sal*I fragment of this cosmid revealed the presence of a complex polymorphism in the *thr*^{BH} allele (D'Andrea et al., 1993), a mutant derived from a hybrid dysgenic cross (Lindsley and Zimm, 1992) (P. Gergen, pers. comm.). The restriction mapping data of the *thr*^{BH} mutant chromosome is consistent with two insertion events, one of an apparently intact P element, and a second of unknown type (Figure 3.1).

To characterise the nature of these polymorphisms in more detail, *Eco*R1 fragments from *thr*^{BH}/*cn bw sp* genomic DNA were cloned into a λgt10 vector (D'Andrea et al., 1993). An *Xba*I-*Sal*I fragment spanning the polymorphic fragments was used to probe for recombinants containing these fragments. A 0.6kb internal *Sal*I fragment was identified as a fragment covering the site of the P element insertion. Although the precise nature of the second lesion in the adjacent *Eco*RI fragment was not determined, Southern hybridisation results were consistent with a non P element insertion generating the novel *Eco*RI fragment of 3.7kb. Genetic analysis revealed that the *thr*^{BH} allele was not revertable in the presence of P transposase. It was not determined whether this non-reversion was due to a defect in the P element insertion or due to an effect of the second insertion on expression of the *thr* gene.

3.2 Isolation of *three rows* coding sequences

As a result of the detection of separate insertional polymorphisms in two flanking genomic *Sal*I fragments of *thr*^{BH} relative to Canton-S, these two fragments were used as probes to isolate cDNA clones from early embryonic libraries (Brown and Kafatos, 1988). The cDNA clones isolated came from three distinct transcription units (Figure 3.1). Restriction mapping and Southern analysis allowed the transcribed regions to be positioned accurately on the molecular map of this region (Figure 3.1).

One transcription unit, defined by cDNA clone UJA8, was shown to span the P element insertion site in *thr*^{BH}, while another, defined by UJB6, coincided with the second non P element insertion event (Figure 3.1). It was not possible at this stage to

determine which of these polymorphisms was the primary *thr* mutational event and hence which of the transcription units represented the best candidate for the *thr* gene.

Two complementary approaches were simultaneously adopted in an attempt to distinguish which transcription unit represented the *thr* gene. Firstly the longest cDNA clones of each transcription unit were subject to sequence analysis. Comparison of the encoded product with extant proteins of known function could either reveal similarities in derived sequence with proteins having demonstrated or conceivable roles in chromosome disjunction, or allow the elimination of one transcription unit based on similarity to proteins having no credible role in mitosis, such as products involved in metabolism.

The respective 4.6 kb and 3.8 kb inserts of cDNA clones UJB6 and UJA8 , were sequenced by assembly of data derived from nested deletions. The sequence of UJB6 was not greatly informative. It did not contain a single extended ORF, and neither its nucleotide sequence nor the products of the short regions of potential coding capacity exhibited significant similarity with any known sequences (data not shown). The sequence of the UJA8 cDNA clone is presented in section 3.4.

The other strategy to unambiguously identify the transcription unit corresponding to *thr* was by genomic complementation.

3.3 Genomic rescue of *three rows* mutants

An 11kb *Not*I genomic DNA fragment, which covered the transcript defined by cDNA clone UJA8, but excluded parts of the neighbouring transcripts (Figure 3.1), was introduced into the *D. melanogaster* germline by P element mediated transformation. Three independent transformants, crossed into a homozygous *thr* mutant background, rescued the *thr* mutant phenotype, confirming that this transcription unit corresponds to the *thr* gene (Figure 3.2)

3.4 Structure of the *three rows* gene

Ten independent *thr* cDNA clones were isolated from a 4-8 h embryonic cDNA library. Curiously cDNA clones were not found in a 0-4 h library, despite the presence of maternal *thr* transcripts (see Chapter 4). In spite of repeated attempts at screening

Figure 3.1 Restriction map of the *grainyhead-three rows* region.

The region deleted in chromosomal deficieny *Df(2R)Pcl 11B* is marked by dashes. Two insertion polymorphisms in the thr^{BH} allele relative to Canton-S from which the cosmid library was derived (J. Tamkun, pers. comm.), one of which was shown to be P element derived, are shown. The position and orientation of the *grainyhead* and *thr* genes are indicated, as well as two cDNA clones UJB6 and UJ4c from transcription units flanking *thr*. The longest *thr* cDNA clones UJA8 and UJE6 are represented. The region spanned by 3 cosmid clones is shown, as well as that of an 11kb fragment, derived from cosmid 3.3a, which was found to be capable of rescuing the *thr* mutant phenotype.

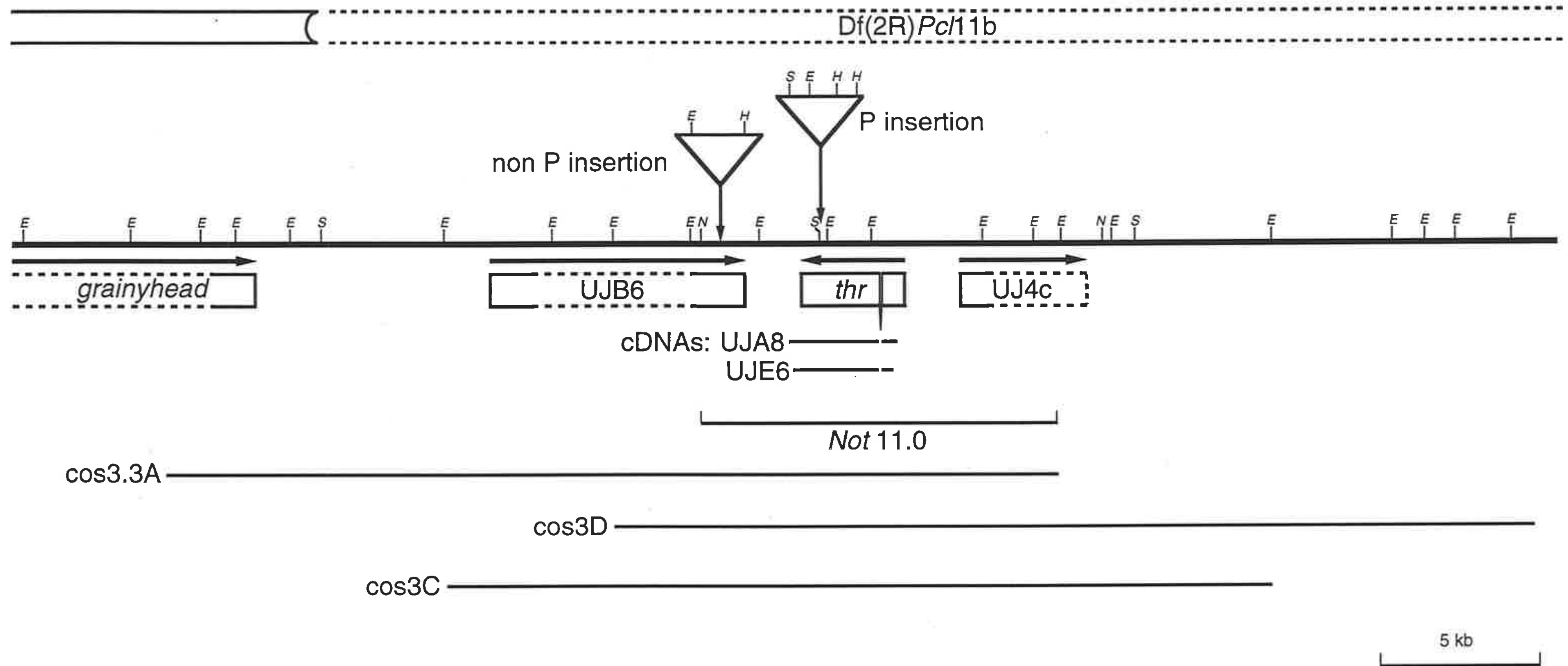

PROXIMAL
DISTAL
Df(2R)Pcl11b
P insertion
non P insertion
grainyhead
UJB6
thr
UJ4c
cDNAs: UJA8
UJE6
Not 11.0
cos3.3A
cos3D
cos3C
5 kb

Figure 3.2 Strategy employed to demonstrate genomic rescue.

Transformant lines carrying the 11.0 kb *Not*I fragment on either the X or 3rd chromosome (crosses for the 3rd chromosome shown here) were crossed to *thr* mutant lines to generate individuals carrying the transformed fragment in a *cn* thr^{313} *bw*/*cn* thr^{IB} *bw* background. Survival of such individuals indicated that the *thr* lethality was rescued by the presence of the 11.0 kb *Not*I fragment. The genotype of viable and phenotypically distinguishable classes of progeny are shown along with their numbers in a typical experiment.

[pCaSpeR4*Not*11.0 + pπ25.7*wc*(Δ2-3)] ⟶ $w^{1118};+$ x $w^{1118};+$

↓

$w^{1118};+;[Pw^{+}]$ x $w;\frac{cn\ thr^{IB} bw}{SM6a}$

↓

$+;\frac{cn\ thr^{313} bw}{CyO}$ x $w^{1118};\frac{cn\ thr^{IB} bw}{+};[Pw^{+}]$

↓

$\frac{cn\ thr^{313} bw}{cn\ thr^{IB}\ bw};[Pw^{+}]$	$\frac{cn\ thr^{313} bw}{+}$	$\frac{cn\ thr^{IB} bw}{CyO}$	$\frac{+}{CyO}$
(*w* since *cn bw*)	(w^{+})	(*Cy*)	
Will only survive if [Pw+] can complement			
Numbers: 11	26	54	

pNB40cDV cDNA libraries for full length cDNA clones, no clone that contained the entire protein coding region was isolated (Figure 3.3). Consequently, the 5' sequence of *thr* was initially obtained from genomic fragments derived from cosmid clones (Figures 3.3 and 3.4). In the latter stages of this study two clones, CT142 and CT151, were obtained from a newly available, random primed 0-18 h embryonic cDNA library, that extended further 5' than the clones obtained from the pNB40 library (Figure 3.3). The assembled cDNA and genomic sequences (Figure 3.4) revealed a large ORF, encoding a predicted 1,379 aa polypeptide. The sequence in the vicinity of the putative initiation codon is in good agreement with the consensus for *D. melanogaster*, including the highly favoured adenine residue at -3 (Cavener and Ray, 1991). An in frame termination codon lies immediately upstream of the presumptive initiation codon (Figure 3.4).

Comparison of the genomic and cDNA sequences in the regions of overlap revealed the existence of two small introns towards the 5' end of the ORF (Figures 3.3 and 3.4). These introns contain consensus splice sequences (Shapiro and Senepathy, 1987) at the junctions with the ORF (Figure 3.4). Although restriction mapping of cDNA and genomic DNA fragments failed to detect any other introns, the existence of other similarly sized introns cannot be excluded.

Comparison of the cDNA clone UJA8 and genomic DNA derived sequences also revealed six nucleotide substitution polymorphisms in the immediate vicinity of the second intron. Three result in changes in the amino acid sequence of the encoded product, two of the changes being conservative (Figure 3.4). These polymorphisms may be the product of error prone replication by reverse transcriptase in construction of the cDNA library. However, the sequence of an additional 7 independent cDNA clones with different 5' termini, derived from the same cDNA library, revealed only a single nucleotide difference (nucleotide 2893, Figure 3.4) in a 2.4kb region of overlap with UJA8. These data, and the silent or conservative nature of 5 of the 6 changes, make it more likely that the polymorphisms observed between the cDNA and genomic DNA sequences reflect true sequence differences between the different strains used to generate the cDNA and genomic libraries.

A 0.6kb *Sal*1 fragment identified previously as defining the site of the P element insertion (D'Andrea et al., 1993) (Section 3.1) was subcloned for sequence analysis. Comparison of this sequence with that of UJA8 revealed that the *thr* ORF was interrupted, in the mutant allele, by the right end of a P element (O'Hare and Rubin, 1983) immediately 5' to nt 3557 (Figures 3.4 and 3.5). The eight nucleotides adjacent to the insertion site show five matches with the consensus derived for P element integration sites (O'Hare and Rubin, 1983)(Figure 3.5). The chimaeric fragment containing the junction between the 5' side of the *thr* ORF and the left P element end was not isolated. However the consequences for the thr^{BH} encoded product if the P element sequence matched that reported (O'Hare and Rubin, 1983) would be premature termination of translation within 3 aa (or 1, or 5 aa for the other two reading frames), and a product 83% of theoretical wild-type length.

Sequence analysis also revealed the existence of alternate polyadenylation sites in *thr* transcripts. Of ten distinct cDNA clones obtained, eight (including UJE6, Figures 3.3 and 3.4) possessed a 111 nt 3' UTR, whilst UJA8 (Figures 3.3 and 3.4) and one other independent clone had a 356 nt 3' UTR. Polyadenylation signals that matched with statistically favoured consensus sequences (Wickens, 1990) were found 51 and 21 nt, respectively, upstream of the poly A tails (Figure 3.4).

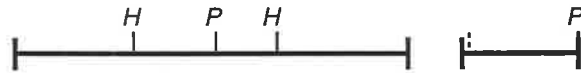
3.5 The *three rows* encoded product

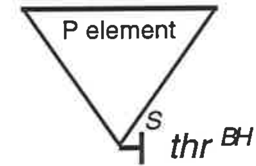
The only large open reading frame derived from the assembled sequence encodes a basic (basic:acidic 211:145, pI=8.62), leucine and serine rich (Table 3.1) protein of predicted M.W. 157,461 Da. A hydropathy profile (Kyte and Doolittle, 1982) suggested that *thr* encodes a protein, with no potential transmembrane domains (results not shown).

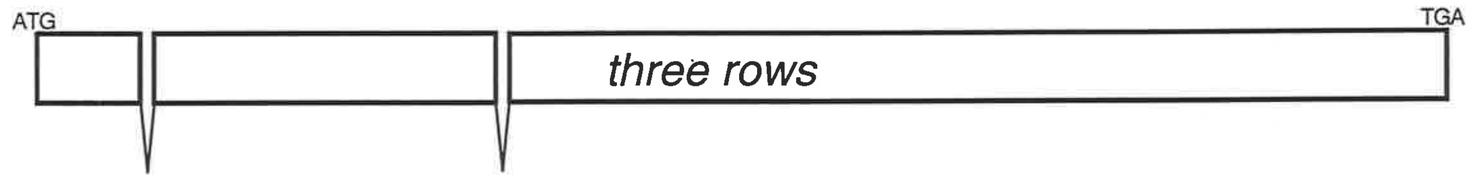
Figure 3.3 The gene structure of *three rows*.

Regions from which genomic sequence was obtained are shown above the gene structure, and the extent of cDNA clones shown below it. Restriction sites: *H Hin*dIII, *P Pst*I, *E Eco*RI, *S Sal*I. A *Pst*I site absent in the genomic sequence is indicated.

Genomic sequence:







cDNAs: UJA8

UJE6

CT142

CT151

```
                                                                             CT142 >
 -112 tttccagctttagcatggacggtcacactggatctcaaaatctggcgcaaaagcaacaaaaaaggaagcgtcgcagttaaactagttaaa

  -22 tgtgccaattccctttgaaaaaATGTCTACTGATATAGCCACCCAGCTGAAGGGCAGCCGCTCCGATGTGGAAAAAGTCCGCAAAACGGT
                            M  S  T  D  I  A  T  Q  L  K  G  S  R  S  D  V  E  K  V  R  K  T  V   23

   69 GGAAGCCAAATTCCGGGAGTTGTCTGGCGATGGACTACCTCTGAGATACGAAGTGAATGTATTGCGCCATATTTGCCTCGCCTTAAAGGA
       E  A  K  F  R  E  L  S  G  D  G  L  P  L  R  Y  E  V  N  V  L  R  H  I  C  L  A  L  K  D   53
                                                                                      HindIII
  159 TAATCTGCACCAGAACTCGGATCTTTACTGCGACATCATGGGCATAATGCTGCCACGCGTAGTTCCCAGCGAGGAGAAGCCAAGCTTATG
       N  L  H  Q  N  S  D  L  Y  C  D  I  M  G  I  M  L  P  R  V  V  P  S  E  E  K  P  S  L  W   83

  249 GGAAGCGCACTTGTCCAGCCTGCGGTACATACATCATGGCTTGTTTCATCAGgtaggagtcgctataagaatcttcgaatctttctaacc
       E  A  H  L  S  S  L  R  Y  I  H  H  G  L  F  H  Q                                          100

  339 aattctcccgttcacagCGATCCATTGAAGCCTGTCAGAAGCTGTACAACCTCATCCGACAACAACCCTGCCGTCTGCAAGAAGAATCAG
                        R  S  I  E  A  C  Q  K  L  Y  N  L  I  R  Q  Q  P  C  R  L  Q  E  E  S  D  125
                                < CT142
  429 ACTACAAAATATATCTGGACATACATTTGACCCACTTCAATGGTTTCCATGTGCTGCTGCAGAAGCAAAAACTCCCCTTGGAAGCTACAA
       Y  K  I  Y  L  D  I  H  L  T  H  F  N  G  F  H  V  L  L  Q  K  Q  K  L  P  L  E  A  T  S  155

  519 GTCAATTGTGTTATGCTTTGGAATCATTGGGAGATCTATTCGCAGCCATGACGCAAAGGCAAATAAGTCTATGCGCAACGCTTCTAGTTC
       Q  L  C  Y  A  L  E  S  L  G  D  L  F  A  A  M  T  Q  R  Q  I  S  L  C  A  T  L  L  V  Q  185
                                                            HindIII
  609 AACTAAACGAGAGTTTATTTGGCAAAAGAAGTAGATCATTCTTCAAGTCCCTAAGCTTCCTGCCGTCGGAGAGTCTTGCCAAAATGTTCA
       L  N  E  S  L  F  G  K  R  S  R  S  F  F  K  S  L  S  F  L  P  S  E  S  L  A  K  M  F  N  215

  699 ACGCGCTGCTTATGCTCCTGGCCAGCAGCACCAGTTCGAACCTGGCCAACTTATTCCCTGAGTGCCTGAGTCTCACGTTGGCTCTCGTAC
       A  L  L  M  L  L  A  S  S  T  S  S  N  L  A  N  L  F  P  E  C  L  S  L  T  L  A  L  V  Q  245
                                UJA8 >
  789 AAATCGACATGTTCAGTCCACAGTCCAATCAACAGATGTCATTGCAGCTGCTGCGCATGAGCAAGGAACTATTTCGCCAGGAATCAAACC
       I  D  M  F  S  P  Q  S  N  Q  Q  M  S  L  Q  L  L  R  M  S  K  E  L  F  R  Q  E  S  N  L  275
                                                                                  UJE6 >
  879 TGTGCTATGCTCTACAGCTGATGTACTACTATATCAAGTTAATCTTTGTTCGGGAACCCACAGGCGACTTTAAGCGCACCTACATTGACT
       C  Y  A  L  Q  L  M  Y  Y  Y  I  K  L  I  F  V  R  E  P  T  G  D  F  K  R  T  Y  I  D  L  305
                                                                              < Genomic
  969 TGTCTAGCAAGTTTCAGCACTTCTTCGAGCACAAAGTAGCCTCGCATGCCAAAGAACAGTGGCTAGCGGATTTTCTGGTGGCCATTCAAT
       S  S  K  F  Q  H  F  F  E  H  K  V  A  S  H  A  K  E  Q  W  L  A  D  F  L  V  A  I  Q  L  335

 1059 TGCTACAGGTGCTCATCCATCAAAGTAACAGTAAGTTGCAGAGTCCTTTTCAGATATTTTGGCAGCAGTTTGACGGAGAGAGCAGTCCCG
       L  Q  V  L  I  H  Q  S  N  S  K  L  Q  S  P  F  Q  I  F  W  Q  Q  F  D  G  E  S  S  P  E  365
                                           Genomic >
 1149 AGATCTACACAGCGCACTTTCAGTTGCTTCAGACGTGTGCTAGCTTGGCGGTTAATATTACGAGGAGTCCTTTGGGCTGCAGTTGCTCCC
       I  Y  T  A  H  F  Q  L  L  Q  T  C  A  S  L  A  V  N  I  T  R  S  P  L  G  C  S  C  S  H  395

 1239 ACGAGGCATGCAAGAGCGTGCGACGGCACTGCATATTGGCGTATGGATTGTGCGCATTAGATGCGTATATTAATTGGAAACCGGCGGCGG
       E  A  C  K  S  V  R  R  H  C  I  L  A  Y  G  L  C  A  L  D  A  Y  I  N  W  K  P  A  A  E  425

          A A
 1329 AGCAGAGAGCAAATGTGgtgggtatttttatctaattttgtggaattttaatgctacatttcaatactattgcagAGCCCTCACAAGCCC
       Q  R  A  N  V                                                              S  P  H  K  P    435
          K  T

      A                     A                                                C
 1419 TTGCTGGGAGTAGTCAAATACTCAATGGATGTGGCTAAGACCATGAAGTGTTTGGGTCCCACCAGTGTGGAGATTATTAAGCTAGTGCGC
       L  L  G  V  V  K  Y  S  M  D  V  A  K  T  M  K  C  L  G  P  T  S  V  E  I  I  K  L  V  R    465
                                                                                 < Genomic
 1509 CAGCTGACATACGTGGCTGATCAGGTCACCTGTCCGGAGCAAATGTCCGTGCTGCTGCCACTTTTGGAGCCACTGCAGAAGCTGCGACCC
       Q  L  T  Y  V  A  D  Q  V  T  C  P  E  Q  M  S  V  L  L  P  L  L  E  P  L  Q  K  L  R  P    495
                                                                                          EcoRI
 1599 TTGGTTGCCGACCAGGATATGAGCAGTTTACTCCGACGCCTCTTTAAGGCCAGCTCCCATTGCGGCGATTCCAATATAGCATGTCGAATT
       L  V  A  D  Q  D  M  S  S  L  L  R  R  L  F  K  A  S  S  H  C  G  D  S  N  I  A  C  R  I    525

 1689 CAAGCTAGTTATTTGGCCTCGATTACGAATCCCGCACGATTAAGATCACAGGTCTGTTTGTACTATCACAATCTGGGAAAAAAGGGCACC
       Q  A  S  Y  L  A  S  I  T  N  P  A  R  L  R  S  Q  V  C  L  Y  Y  H  N  L  G  K  K  G  T    555

 1779 GAGATCAAAAGGTGTGTCTACGAGTGGCACGAGTCCACGCCACTACCTTTCCCTCTCACTCCGGACCAGAAGAAACAGCTGTATGATACC
       E  I  K  R  C  V  Y  E  W  H  E  S  T  P  L  P  F  P  L  T  P  D  Q  K  K  Q  L  Y  D  T    585

 1869 GATTTCTTTGCCTTACTACACTATTTGAGGAGTCCTTCTACGGCTCATATGGAATCACTTATTCGTTGCCGAACGAGTGACTATCATCTG
       D  F  F  A  L  L  H  Y  L  R  S  P  S  T  A  H  M  E  S  L  I  R  C  R  T  S  D  Y  H  L    615

 1959 GTACTCTTGGCCAGACAAATGCGAAAGGATGACTCGATTTCGAAGAAATGCATAGAGGTTCATGATAAGCTAAGGCAACAACGTTCGCTC
       V  L  L  A  R  Q  M  R  K  D  D  S  I  S  K  K  C  I  E  V  H  D  K  L  R  Q  Q  R  S  L    645

 2049 AGTCGAATGGATAACTTGTGCCTGGGCCACGCAAGTGTGGGACTACTACTGGACGCACTGGAGGCTCAAAAAACCAAAGTTTCCACCAAG
       S  R  M  D  N  L  C  L  G  H  A  S  V  G  L  L  L  D  A  L  E  A  Q  K  T  K  V  S  T  K    675

 2139 GAGATAACGGAAAATATGTTCGAGGAGCTGCTACTCAGCAAGAATTTATGGCAGATGAACATACAAAGGGAGCAGCGATTGGTCAACTAT
       E  I  T  E  N  M  F  E  E  L  L  L  S  K  N  L  W  Q  M  N  I  Q  R  E  Q  R  L  V  N  Y    705

 2229 GCTAGTGAAGCCATCTCGGCCTTCAGCAACTTCTTCGATCGAGCAGATCAAGAGCCATTGAGCGCAAATGAAACGTCTATTGATTGGGAG
       A  S  E  A  I  S  A  F  S  N  F  F  D  R  A  D  Q  E  P  L  S  A  N  E  T  S  I  D  W  E    735

 2319 GCATTGATTGACGATGCCATCGCTACTGCCAATGCACTTTCAAGTATGGGGTATCAGTCAGAAGAGGATGATGCCTGGCTGTTGCTTCTG
       A  L  I  D  D  A  I  A  T  A  N  A  L  S  S  M  G  Y  Q  S  E  E  D  D  A  W  L  L  L  L    765

 2409 AGGATGGGTCGCTTGCTGGAAGATCGTTTTACCTATCTGCGTGCCCTAAATCATTTTCTGTCACAGAATGAGGTTAGTTCTCGTTTAAAT
       R  M  G  R  L  L  E  D  R  F  T  Y  L  R  A  L  N  H  F  L  S  Q  N  E  V  S  S  R  L  N    795

 2499 CTGAAACTCGGCGAGGAAGTGGAAGTAGCAGAGGAATTGCTGGATGATTTGTGGCCTCAATTGAAAAATGGCAAATTCTTCAAGCGTCAG
       L  K  L  G  E  E  V  E  V  A  E  E  L  L  D  D  L  W  P  Q  L  K  N  G  K  F  F  K  R  Q    825

 2589 CAAACTACGGTAATGCTCTGTTTTTGTCACCTCGCCAGTTACTATGCCAGAATGGAATGCTATAGTCATGCCCAGTTGCTTCTATTGCAT
       Q  T  T  V  M  L  C  F  C  H  L  A  S  Y  Y  A  R  M  E  C  Y  S  H  A  Q  L  L  L  L  H    855

 2679 GTGGAACAACTTCGCGAAGAGTTTCCTGAGAGACAAGGAAAAAGTGATATTGTATTGCTTACACTGCAAACGGTGCGCTTTCGAATTGGG
       V  E  Q  L  R  E  E  F  P  E  R  Q  G  K  S  D  I  V  L  L  T  L  Q  T  V  R  F  R  I  G    885

 2769 TATCAGCAAAGGAAGCCAACGAATTGCAGGCTGCCGACTCCTCTGCGTCAATTGGACATTCTTCTGGACAATGTGCGAAGTTTTTGCAAT
       Y  Q  Q  R  K  P  T  N  C  R  L  P  T  P  L  R  Q  L  D  I  L  L  D  N  V  R  S  F  C  N    915
```

```
                                       T (UJA9)
2859 CTATCCAGTTTAGATGGCGGCTCATTGCAGCTGCCTCTTTCGACTCTTGTCAGGGAAAGCACCGAGTCGTCTGCGAACAGATTAAGCGAA
     L  S  S  L  D  G  G  S  L  Q  L  P  L  S  T  L  V  R  E  S  T  E  S  S  A  N  R  L  S  E    945
                                      L

2949 AGACTGTCCTTCTCCAACATTGCACTACATTTGGTACTCCAATCTGGCTTGGCTTTAAGAGCCATAGAGGTATTCCTTGCATGGTTATGG
     R  L  S  F  S  N  I  A  L  H  L  V  L  Q  S  G  L  A  L  R  A  I  E  V  F  L  A  W  L  W    975

3039 ACCAATTTGCAAATGGAAAGTTTCGACAAGGCGCAATCGAAGTTGAGACTCATCGAGCATTGTTTAGGTATAAAACAGCTGAATCCAACG
     T  N  L  Q  M  E  S  F  D  K  A  Q  S  K  L  R  L  I  E  H  C  L  G  I  K  Q  L  N  P  T    1005

3129 AGTAGGCCGGAAAAGGAAGCGATTAAGGATGTAGCAATTAGTGATCTGGCTAGTAATATGCATCTCCTCCAATTGGTGGAGCCGATCAGG
     S  R  P  E  K  E  A  I  K  D  V  A  I  S  D  L  A  S  N  M  H  L  L  Q  L  V  E  P  I  R    1035

3219 AAGCAGCAGCTATTAAATATGGCCTCGCCGAATCTGCTCAAAATGCGACCACATAGCCCAAATCCCCAACTGGACTTGGATCGCTACATA
     K  Q  Q  L  L  N  M  A  S  P  N  L  L  K  M  R  P  H  S  P  N  P  Q  L  D  L  D  R  Y  I    1065
                                     EcoRI
3309 ACACTCGATGTGGCGCCAGCGAATCTTCGAGAGAATTCCCAGCTGCAATGCTTGTACTTTGTAACGGGCTGTCTACATGCACGTCTCCGC
     T  L  D  V  A  P  A  N  L  R  E  N  S  Q  L  Q  C  L  Y  F  V  T  G  C  L  H  A  R  L  R    1095

3399 TTTCTGCAGAGAAACAGCGAACAATTGGAGGAGTTCTATGGAAGAGCGCATAACTGGATGCAGGAAAAACCTCCGATGAGTAGCGCTTTG
     F  L  Q  R  N  S  E  Q  L  E  E  F  Y  G  R  A  H  N  W  M  Q  E  K  P  P  M  S  S  A  L    1125
                                                                        < P element
3489 TATCCCATGTTGCATGCCCAGCAGCTGTATCATCTCAACTATCTTCGCTTTGCGCGAAAGCATGTAGAGGCTATATCAACGGCTCAATTG
     Y  P  M  L  H  A  Q  Q  L  Y  H  L  N  Y  L  R  F  A  R  K  H  V  E  A  I  S  T  A  Q  L    1155
                             SalI < BH14 Genomic
3579 GGCCTTAAAATGCGATCACGCGCGGTCGACATTAATTTCGAGTACAATTTTTTGGCTCAGCTAAAGACGGCTCAGTTAGAACTCAAACCC
     G  L  K  M  R  S  R  A  V  D  I  N  F  E  Y  N  F  L  A  Q  L  K  T  A  Q  L  E  L  K  P    1185

3669 GTGGGCCAGGATAAGCCACAGGTCAAAATCCTTCGACGTGCTTTGGTATTCAATCACTCACCCGAAGATAAGAAACGAACCGCAACAGGA
     V  G  Q  D  K  P  Q  V  K  I  L  R  R  A  L  V  F  N  H  S  P  E  D  K  K  R  T  A  T  G    1215

3759 TCGGTTTCAGCAGTCAAGAATACGGCGTCTAAAGTTAAACAGTCGGCCAAAAAGGCACCTCGATTCAGAATTTACGAGGAGCTGGAACTA
     S  V  S  A  V  K  N  T  A  S  K  V  K  Q  S  A  K  K  A  P  R  F  R  I  Y  E  E  L  E  L    1245

3849 CGACCACCAAGTGCTACCAGTTGCAGTAGCAGCGGTGGCAGCGGAACAGAGAATACACCGCCTTCGGATCACGTGGATCTAAATGCCTGC
     R  P  P  S  A  T  S  C  S  S  S  G  G  S  G  T  E  N  T  P  P  S  D  H  V  D  L  N  A  C    1275

3939 CAAGCGATCGAGATAAGCGACGATGACGATTCACCTTTGGTGTCCACAAAGAAGACACAACCAAAAAGTAGGGAGAAGGCAAAACCCAAG
     Q  A  I  E  I  S  D  D  D  D  S  P  L  V  S  T  K  K  T  Q  P  K  S  R  E  K  A  K  P  K    1305
               HindIII
4029 GCCACATCCAAAGCTTGTAAAGTCCTAACATTGGATAATAGCTTGGAAATAGTAGAAACGCCGACAATAACTACAAGTACACGGAGCACC
     A  T  S  K  A  C  K  V  L  T  L  D  N  S  L  E  I  V  E  T  P  T  I  T  T  S  T  R  S  T    1335

4119 AGAGCCAGGCTGCGCCAACCAGTAGAGACACCAAAGACAGCGACTCTTTCATCCAAGCGAACGAGGCGCCAGGTTTTGGAAGCACAGGCT
     R  A  R  L  R  Q  P  V  E  T  P  K  T  A  T  L  S  S  K  R  T  R  R  Q  V  L  E  A  Q  A    1365

4209 CCCGAAACGGAGTCCATTAGCACACGCACACGGCATCGGCACTGAttagatagtagtctccttagttcgtatttattcttatcgaaaatg
     P  E  T  E  S  I  S  T  R  T  R  H  R  H  *  1379
                                                          UJE6 (A)n >
4299 tcttggtatttaaatatatatatatattgtgtgtaagtgttacaaaagagcttttgacataaattcattgtttttatcttgtggcaggg

4389 acagtagtgacgttctacagatgtccttgctccaaaaaccgtgctgaacccgctgtccaattgctggcgtgggtaaaatacacctgagcc

4479 tgatttgcagcctccttcaaattgacccacgtagcgatttcccgttgctatggataacatattaacttattatctataatctttatgcat
                                 UJA8 (A)n >
4569 ttttaaagttgcgaaaaaataaataaaatgaaacaaat 4606
```

Figure 3.4 Nucleotide sequence and derived amino acid sequence of *three rows*.

The nucleotide sequence of the *three rows* gene contains a long open reading frame encoding a theoretical protein of 1,379 amino acids. Coding sequence is shown in upper case, and 5', intron and 3' noncoding sequence is shown in lower case. An in frame termination codon, 30 nt upstream of the putative intiation codon, is shown in bold. *Hin*dIII, *Eco*RI and *Sal*I restriction sites are underlined. Intron splice donator and acceptor sites are shown in bold. The derived amino acid sequence is represented in one letter code below the nucleotide sequence. The 5' termini of the cDNA clones, CT142, UJA8 and UJE6, are shown with arrowheads (the position of the arrowhead marks the first nucleotide). The extent of nucleotide sequence derived from subcloned fragments of cosmid 3.3a, and from CT142 is delineated by arrowheads. Six nucleotide substitution polymorphisms in the genomic sequence, relative to the cDNA sequence, are shown above the cDNA sequence. The resulting amino acid substitutions are shown below the cDNA amino acid sequence. A further nucleotide substitution polymorphism, and consequent amino acid sequence difference, detected in cDNA clone UJA9 is indicated. Nucleotide sequence derived from the dysgenic allele thr^{BH} extends from the arrowhead marked "*BH14* Genomic" to the position indicated as "P element" where the cDNA sequence is interrupted by a consensus P element right end (O'Hare and Rubin, 1983) (Figure 3.5). The nucleotide at which poly A tails commence in cDNA clones UJA8 and UJE6 is arrowed in the 3' untranslated sequence. Putative polyadenylation signals are shown in bold. A region of amino acid sequence with similarity to part of the product of the *nuc2* gene of *S. pombe* (Figure 3.6) is underlined.

Figure 3.5 Alignment of sequence at the junction of P element and the 3' end of the *thr* ORF in thr^{BH}, with corresponding regions in UJA8 and pπ25.1.

* From O'Hare and Rubin (1983).

```
Consensus 8 nt sequence
adjacent to P element*:                        GGCCAGAC
                                               ||| | |
UJA8:     3525 AACTATCTTCGCTTTGCGCGAAAGCATGTAGAGGCTATATCAACGGCTCAATTGGGCCTTAAAATGCGATCACGCGCGGTCGAC 3608
                                               ||||||||||||||||||||||||||||||||||||||||||||||||||||
thrBH:         CCGACGGGACCACCTTATGTTATTTCATCATGGGCTATATCAACGGCTCAATTGGGCCTTAAAATGCGATCACGCGCGGTCGAC
               ||||||||||||||||||||||||||||||||                                              SalI
pπ25.1*:  2876 CCGACGGGACCACCTTATGTTATTTCATCATG 2907
```

Table 3.1 Comparison of percentage amino acid content of three rows with that of the average in proteins.*

Amino acid	three rows	average	difference
A	7.1	8.6	-1.5
G	2.7	8.4	**-5.7**
L	14.8	7.4	**+7.4**
S	9.5	7.0	**+2.5**
V	4.5	6.6	-2.1
K	5.7	6.6	-0.9
T	4.9	6.1	-1.1
E	6.2	6.0	+0.2
D	4.3	5.5	-1.2
P	4.4	5.2	-0.8
R	6.5	4.9	+1.6
I	4.1	4.5	-0.4
N	3.8	4.3	-0.5
Q	6.4	3.9	**+2.5**
F	3.6	3.6	0
Y	2.8	3.4	-0.6
C	2.5	2.9	-0.4
H	3.0	2.0	+1.0
M	2.2	1.7	+0.5
W	0.9	1.3	-0.4

* according to Dayhoff (1978)

Inspection of the derived *thr* product revealed consensus motifs implicated in a number of protein activities or modifications. Some of these are shown in Table 3.2. Given the basic nature of the encoded product, particularly at the C-terminal end, the occurrence of 3 potential nuclear targeting sequences is not surprising. In addition to those shown in Table 3.2, three rows also contains many potential phosphorylation sites for cAMP/cGMP dependent protein kinases, protein kinase C, calcium/calmodulin dependent protein kinase, and casein kinases I and II (Kennelly and Krebs, 1991). The biological significance, of these phosphorylation sites, and the leucine zipper motifs (Table 3.2) are somewhat

dubious. There are clearly other determinants to protein kinase activity than the amino acid residues surrounding a phosphorylation site (Kennelly and Krebs, 1991). Consensus leucine zippers are known to occur in proteins that are neither transcription factors nor undergo dimerisation (GCG Prosite program notes), and their fortuitous occurence in a protein as leucine rich as three rows (Table 3.1) is not unexpected. The putative nuclear targeting sequence with the the best match to the consensus (Dingwall and Laskey, 1991), at residues 1197-1211, is coincident with motifs (Kennelly and Krebs, 1991) for phosphorylation by cAMP/cGMP dependent protein kinases, protein kinase C, and casein kinase II (Table 3.1).

Table 3.2 Putative sequence motifs in three rows.

Activity	Consensus	Position	Sequence
Nuclear targeting sequence[1]	$(R/K)_2$-X_{10-12}-$(R/K)_{3/5}$	1144-1162	**RK**HVEAISTAQLGL**K**M**R**S**R**
		1197-1211	**RR**ALVFNHSPED**KKR***
		1292-1305	**KK**TQP**K**S**R**E**K**A**K**P**K**
Cyclin Dependent Protein Kinase Phosphorylation Site[2]	S/T-P-X-R/K	431-434	**SP**H**K**
		898-901	**TP**L**R**
Tyrosine Kinase Phosphorylation Site[3]	R/K-X_{2-3}-D/E-X_{3-2}-Y	119-126	**R**LQ**E**ESD**Y**
		769-777	**R**LL**E**DRFT**Y**
Leucine Zipper[4]	L-X_6-L-X_6-L-X_6-L	30-51	**L**SGDGLP**L**RYEVNV**L**RHICLA**L**
		764-785	**L**LRMGRL**L**EDRFTY**L**RALNHF**L**
		1083-1104	**L**YFVTGC**L**HARLRF**L**QRNSEQ**L**

*Motifs matching the consensus[1] for Casein Kinase II, cAMP/cGMP dependent protein kinase and Protein Kinase C are located at residues 1205-1208, 1209-1212 and 1209-1212 repectively.

References:
[1] (Dingwall and Laskey, 1991)
[2] (Kennelly and Krebs, 1991)
[3] (Cooper et al., 1984)
[4] (Landschulz et al., 1988)

The *thr* encoded product also contains 4 regions (3 at the C-terminal end) which give high scores with the algorithm for identifying PEST sequences (Rogers et al., 1986) (Table 3.3). PEST sequences are regions rich in proline, glutamic acid, aspartic acid, serine and threonine, flanked by basic amino acids, that are present in many unstable proteins.

Table 3.3 PEST sequences in three rows.

Position	Sequence	PEST-FIND score*
565-579	HESTPLPFPLTPDQK	+9.4
1246-1269	RPPSATSCSSSGGSGTENTPPSDH	+14.9
1312-1333	KVLTLDNSLEIVETPTITTSTR	+2.7
1358-1374	RQVLEAQAPETESISTR	+2.5

*Calculated as described (Rogers et al., 1986). Scores can range from +45 to -45.

Comparisons with sequence databases using the program FASTA (Pearson and Lipman, 1988) revealed no extended sequence similarity to any known protein. By contrast, the program BLAST (Altschul et al., 1990), which optimises matches between short motifs, indicated that a sequence at residues 705-729 of the encoded product exhibited 44% identity (smallest Poisson probability 0.015) with residues 220-244 of the product of the *nuc2* gene of *S. pombe* (Figure 3.6). *nuc2* encodes a 665 aa TPR protein with an essential role in mitosis (Hirano et al., 1988) (Section 1.3.2). The possible significance of this similarity is discussed below.

3.6 Discussion

The *thr* gene has been identified in a chromosomal walk from the neighbouring *grh* gene, by association with a P element insertion event in the dysgenic allele thr^{BH}. Isolation has been confirmed by P element mediated germ line transformation of an 11 kb

genomic fragment, capable of complementing embryonic lethality when present in a homozygous *thr* mutant background. Sequence analysis of cDNA and genomic clones have revealed a large ORF encoding a predicted 1,379 aa polypeptide. During the course of this study *thr* was cloned independently using a different approach (Philp et al., 1993). In contrast to the findings of the present study Philp et al. (1993) were able to revert the thr^{BH} dysgenic allele. The sequence reported encodes a 1172 aa product that can be resolved with the 1379 aa reported here by allowance for the 5' most intron and a frameshift near the 3' end of the ORF. The frameshift results from a single extra nucleotide in the sequence presented here (a C at 4133, Figure 3.4), found in a region of sequencing gel "compression". The sequence in this region has been confirmed by "dITP" sequencing on both strands (data not shown), and furthermore is consistent with that of a *thr* homologue (see Chapter 6). The results of Philp et al. (1993) also reveal the existence of yet another polyadenylation site 82 nt downstream of the termination codon reported here.

Analysis of the derived product of the *thr* gene does not give any clear indication of its precise function in chromosome disjunction. Three rows contains motifs that suggest it may be nuclear localised and could be unstable. The coincidence of phosphorylation sites with a nuclear targeting sequence is provocative, in the light of findings that implicate protein phosphorylation in modulating the efficiency of targeting (Moll et al., 1991; Rihs et al., 1991).

Searches of the sequence data bases show the derived protein has no extended sequence similarity with any known protein. Three rows is thus unlikely to be directly responsible for chromosome movement, having no consensus NTP binding sites, and exhibiting no homology to any known motor protein. One potential clue to the function of the protein is a very small region, with apparently significant similarity to a sequence outside the TPR domains, in the $p67^{nuc2}$ protein of *S. pombe* (Figure 3.6). *nuc2* is required for chromosome disjunction although, unlike *thr*, mutants appear to undergo a true cell cycle arrest in a metaphase like stage (Hirano et al., 1988). However it is difficult to conceive that this region of similarity is a product of functional conservation during evolution, as the motif is not found in the *nuc2* homologues in *S. cerevisiae* or *A. nidulans*

Figure 3.6 A region of amino acid sequence similarity between the *three rows* encoded product and the *S. pombe nuc2* product.

Identical residues are boxed and conservative substitutions using the following groupings: A, L, V, I, M; K, R; D, E; S, T; N, Q; Y, F, are circled. The derived *nuc2* sequence is from Hirano et al. (1988).

S. pombe nuc2	220	Y	S	N	S	S	I	S	A	F	T	K	W	F	D	R	V	D	A	S	E	L	P	G	S	E	244
D. melanogaster thr	705	Y	A	S	E	A	I	S	A	F	S	N	F	F	D	R	A	D	Q	E	P	L	S	A	N	E	729

(Section 1.3), nor indeed in those recently isolated from humans and *D. melanogaster* (Tugendreich et al., 1993; Lamb et al., 1994).

It would therefore appear that three rows is a "pioneer" protein which defines an as yet uncharacterised activity required for chromosome disjunction. The function of three rows is further addressed in subsequent chapters.

Chapter 4 (Prometaphase): Analysis of expression

4.1 Background

The *thr* mutant phenotype is especially noteworthy for its embryonic lethality. As described in section 1.5.3 the maternal contribution of most components required for proliferation appears to be sufficient for all the embryonic cycles in *D. melanogaster*. Defects in embryonic proliferation with consequent embryonic lethality result from mutations in genes encoding essential cell cycle functions if the maternal contribution is insufficient to provide for the entire set of embryonic cell cycles. This is true in particular for the products of genes encoding regulatory functions such as *string* and cyclins A and E (Sections 1.5.3.3 and 1.5.4) since cell cycle progression is dependent on their periodic synthesis and degradation.

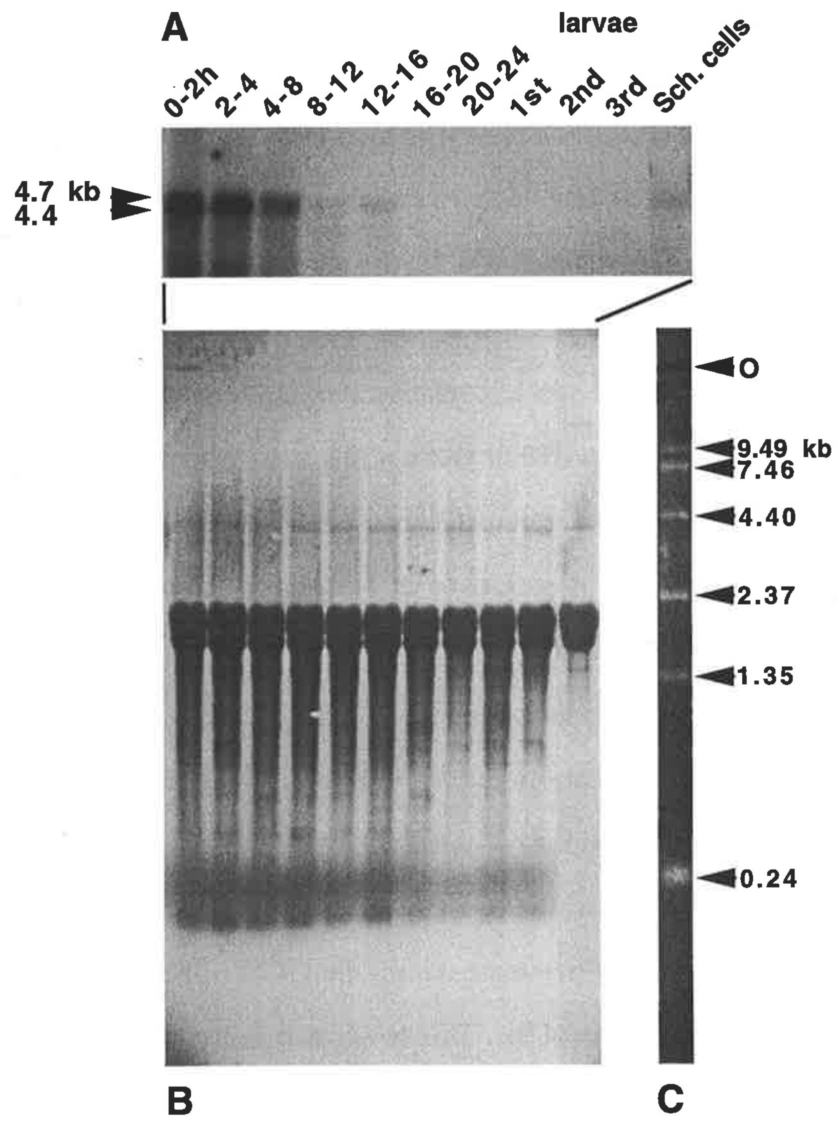
The aim of this work was to characterise the expression of *thr*. In addition to determining the number and length of transcripts, it was intended to establish the temporal pattern of *thr* transcription in relation to both the cell cycle, and development. These data would be used to: determine if levels of *thr* message exhibited cell cycle dependence as would be expected if it had a regulatory function in mitosis; interpret the cycle 15 defect observed in homozygous mutant embryos; and ascertain the role of *thr* in proliferation at other (non-embryonic) stages of development.

4.2 Northern analysis

Hybridisation of a *thr* cDNA probe to a developmental Northern blot of total RNA revealed high levels of *thr* transcripts in embryos up to 4 h post fertilisation and reduced levels up to 16 h of embryonic development (Figure 4.1). Northern analysis was unable to detect transcripts late in embryogenesis, during larval development (Figure 4.1), or in pupae or adults (data not shown), but transcripts were clearly present in *D. melanogaster* Schneider line 2 cultured cells (Figure 4.1).

Figure 4.1 Detection by Northern analysis of *three rows* transcripts during development of *D. melanogaster*.

A. Northern blot of total RNA isolated from timed collects of embryos and larvae, and Schneider line 2 tissue culture cells, hybridised with the UJA8 cDNA as probe. Two hybridising bands corresponding to 4.4 and 4.7 kb transcripts were apparent on shorter autoradiographic exposures, but are obscured in the long exposure shown here to illustrate the levels of *thr* mRNA during embryonic and larval development. B. Membrane stained with methylene blue to illustrate relative levels of RNA loaded. C. Aligned Ethidium bromide stained track of BRL RNA size markers.



A
larvae
0-2h
2-4
4-8
8-12
12-16
16-20
20-24
1st
2nd
3rd
Sch. cells
4.7 kb
4.4
O
9.49 kb
7.46
4.40
2.37
1.35
0.24
B
C

In some exposures it was possible to resolve two species of transcript of approximate size 4.4 and 4.7 kb (Figure 4.1 and data not shown).

4.3 RNase protection analysis

RNase protection experiments using an antisense RNA probe derived from the *Hin*dIII fragment at nucleotides 240 to 666 (Figure 3.4) confirmed the findings of the Northern analysis but the increased sensitivity also enabled the detection of *thr* transcripts in third instar larvae, adult males and females (Figure 4.2). These results reinforce the correlation between *thr* expression and cell proliferation in all tissues. Transcripts present in adult females presumably include maternal *thr* transcripts generated during oogenesis (Section 4.5).

Although precise size determinations are impossible without RNA size standards the mobility of the protected species, relative to a DNA sequence ladder (data not shown), is consistent with the 310 nt product that would be expected to derive from sequences 3' to the first of the introns (Figure 3.4). The presence of probe sized product in some gel tracks (Figure 4.2) is indicative of incomplete RNase digestion.

4.4 Primer extension analysis

A primer complementary to the *thr* ORF at nucleotides 475 to 494 (Figure 3.4) was employed to elucidate the position of transcription initiation. This revealed the presence of products with 5' termini at positions (relative to numbering in Figure 3.4) of: 380±1, 96±2 (if genuine both predicted to terminate within the ORF), -58±5 (predominantly in early embryos) and -190±30 (Figure 4.3). This result was reproducible and observed when the extension reaction was performed in the presence of Actinomycin D, to reduce secondary structure formation, and hence inhibit the production of anomolous extension products due to polymerase pausing. The position of the priming site chosen, with respect to the 5' end of the gene, was based on incomplete sequence information obtained early in this study. In retrospect it would have been desirable to have utilised an oligonucleotide that primed nearer the initiation codon, thus enhancing the yield of full length extension products.

The molecular basis for the variant lengths of cDNA extension products has not been determined. They may derive from transcripts that arise from initiation at different sites, or from the processing of a single primary transcript, such as by intron excision. Two of the major products detected, if specific, are predicted on the basis of sequence data (Figure 3.4) to terminate within coding sequence. However it is possible that these products are not *thr* specific, as their abundance in 8 to 24 h embryos is inconsistent with levels of *thr* transcript detected by other methods of expression analysis employed here. Conversely, they may be authentic and could, for example, arise from the excision of additional introns, not detected in the limited range of cDNA clones decribed in Chapter 3. The second longest primer extension product is estimated to terminate as little as 10 nucleotides upstream of the 5' end of the cDNA clone CT142 (Figure 3.4). In an independent study (Philp et al., 1993) a *thr* transcript has been identified, by RACE PCR, starting at -722±5 (their numbering) which appears to correspond to the longest primer extension product identified here. Inspection of upstream sequences (this study and Philp et al., 1993) has failed to detect "TATA" motifs, approximately 30 nt upstream of any of the identified 5' termini.

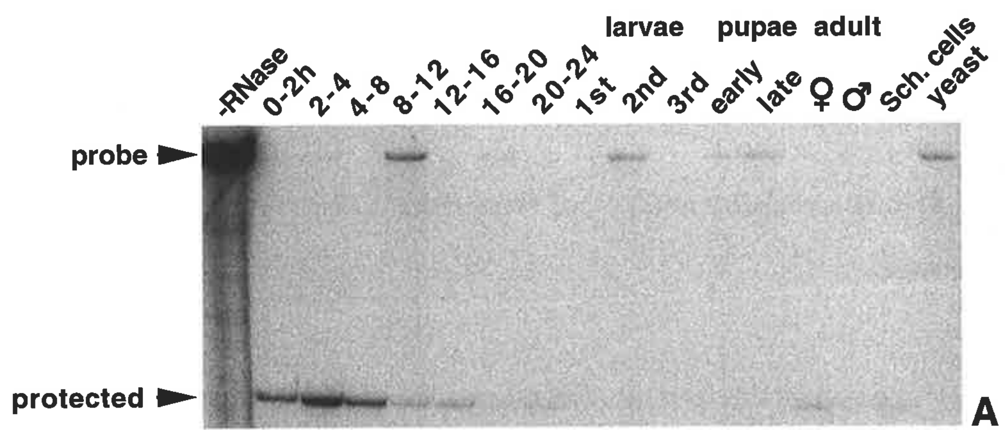
The two longest 5' UTR lengths detected, in combination with the alternate 3' lengths identified in the cDNA clones (Figure 3.4) can notionally give rise to transcripts between 4.3 and 4.7 kb in length (excluding the poly(A) tail), which is consistent with those sizes estimated from the Northern (Figure 4.1).

4.5 Whole mount *in situ* hybridisation to mRNA

To characterise the spatial and temporal distribution of *thr* mRNA during embryonic development, digoxigenin labelled cDNA, derived from clone UJA8, was hybridised *in situ* to fixed embryos (Figure 4.4). Throughout the syncytial divisions, prior to cellularisation, embryos exhibited high levels of *thr* transcripts (Figure 4.4A). In these embryos, the *thr* transcripts appeared to be concentrated around the nuclei. A similar distribution has been observed for transcripts of cyclin B (Lehner and O'Farrell, 1990b) and shown to be dependent on microtubule integrity (Raff et al., 1990) and sequences in the 3' UTR (Dalby and Glover, 1992) (Section 1.5.4).

Figure 4.2 Detection by RNase protection analysis of *three rows* transcripts during development of *D. melanogaster*.

Product protected from digestion by RNase when a 421 nt RNA probe is hybridised to total RNA isolated from life cycle stages and Schneider line 2 tissue culture cells, with yeast RNA as a control. The results of two independent experiments are shown (A and B), with a longer autoradiographic exposure (B) also revealing message in third instar larvae and adult males. The position of the undegraded RNA probe and the protected species is indicated.


larvae
pupae
adult
-RNase
0-2h
2-4
4-8
8-12
12-16
16-20
20-24
1st
2nd
3rd
early
late
♀
♂
Sch. cells
yeast
probe
protected
A

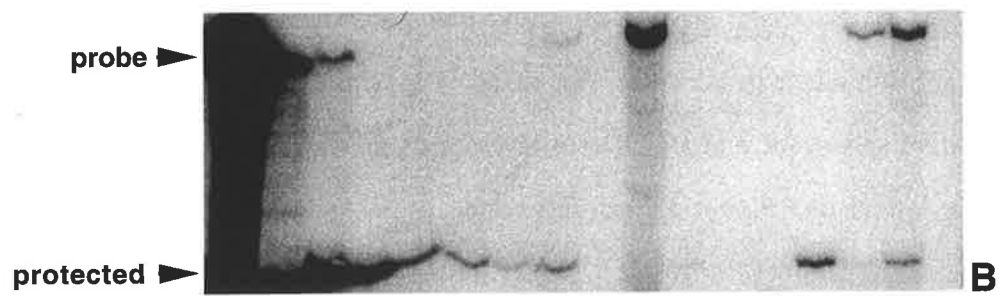

probe
protected
B

Figure 4.3 Primer extension analysis of *three rows* transcripts during development of *D. melanogaster*.

Resolution of oligonucleotide primed cDNA extension products from total RNA isolated from: 1. 0-2h, 2. 2-4h, 3. 4-8h, 4. 8-12h, 5. 12-16, 6. 16-20h, 7. 20-24h embryos, 8. 1st, 9. 2nd, 10. 3rd instar larvae, 11. early, 12. late pupae, 13. adult females, 14. males, 15. Schneider line 2 cells, 16. yeast. The results of two independent experiments are shown (A and B). The mobilities of the extension products relative to the sequence ladder and Figure 3.4 are indicated.

-190±30
-58±5
(96±2)
(380±1)

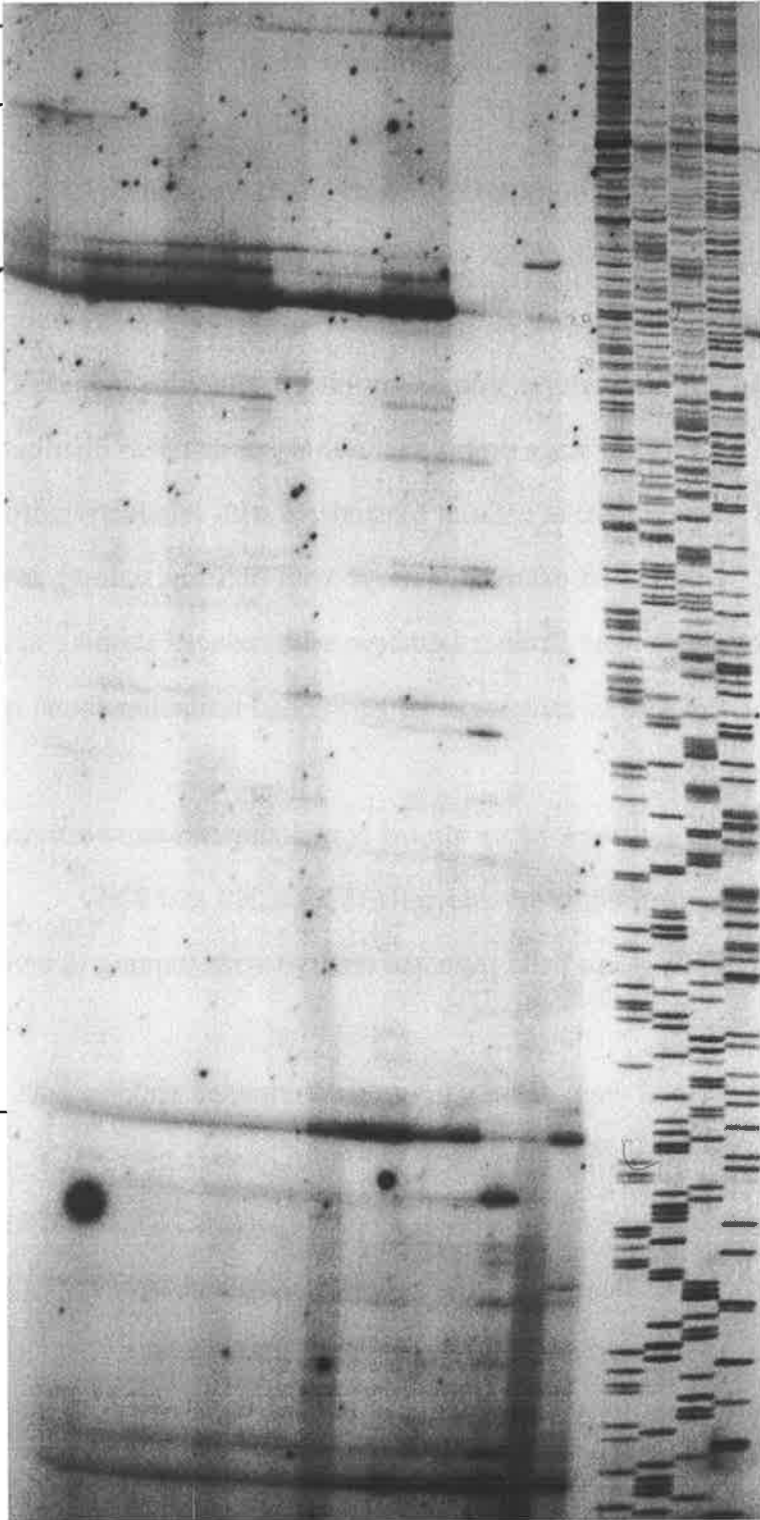


Figure 4.4 Detection of *three rows* transcripts during development of *D. melanogaster* by *in situ* hybridisation of digoxigenin labelled probes to wild type and *three rows* deficient embryo whole mounts.

Whole mount embryos were hybridised with the UJA8 cDNA labelled by incorporation of digoxigenin-11-dUTP. Hybridisation was detected using an alkaline phosphatase coupled secondary antibody detection system. All embryos are orientated with anterior to the left and ventral side down unless otherwise indicated.

A. Syncytial stage embryo exhibiting perinuclear distribution of transcript.

B. An embryo at cellular blastoderm with relatively uniform message distribution.

C. Germ band extended embryo with uniform staining in all but the amnioserosa.

D. Germ band extended embryo with reduced staining in all but cells of the CNS.

E. Embryo in early stage of germ band retraction showing expression in cells of the CNS and PNS.

F. Ventral view of an almost complete germ band retracted embryo showing transcripts in segmentally reiterated cells of the CNS and PNS.

G. Fully germ band retracted embryo with staining in ventral nerve cord and brain lobe (CNS).

H. Dorsal view of fully germ band retracted embryo with transcripts confined to cells in the proliferating margins of the brain lobes.

I, J, K: Progeny of *Df(2R)PC4/CyO* parents. *Df(2R)PC4* removes *thr* in its entirety. One quarter of the embryos at cellularisation and later stages were lacking *thr* transcripts.

I. An embryo during cellularisation showing lack of transcripts in all cells except pole (presumptive germ) cells.

J. Cellular blastoderm at higher magnification showing punctate staining in pole cells.

K. Early germ band extended embryo with transcripts restricted to the pole cells.

A

B

C

D

E

F

G

H

I

J

K

Following cellularisation, levels of *thr* transcripts were seen to remain high and evenly distributed, except for a higher concentration in the region of the pole cells (Figure 4.4B). During germ band extension, transcript levels were high except in the amnioserosa (Figure 4.4C), but declined during cycles 15 and 16 (Figure 4.4D) to be undetectable in most tissues prior to germ band retraction. Significantly, there was no indication of any stage-specific regulation of *thr* transcript levels during cycle 15. At the germ band extended stage and the early stages of retraction, *thr* expression was clearly observed in cells of the CNS and PNS (Figure 4.4E, F). Following germ band retraction, *thr* transcripts were confined to a subset of cells within the ventral nerve cord and in the proliferative centres of the brain lobes, (Figure 4.4G, H). These are the only proliferating tissues at this stage of development. Expression was not detected in cells undergoing endo-replication cycles, cycles of DNA replication that proceed in the absence of mitosis to generate polytene chromosomes (Smith and Orr-Weaver, 1991).

Levels of *thr* message appear to be relatively stable during embryogenesis and do not fluctuate in an obvious cell cycle dependent manner. In the progressive snapshots of development represented in collections of fixed embryos, the pattern of cells in which *thr* message was detected did not undergo marked changes. This is in contrast to the temporally and spatially dynamic patterns of expression observed for established cell cycle regulators such as *string* (Edgar and O'Farrell, 1989) and cyclin E (Richardson et al., 1993).

To follow the fate of the maternal contribution during embryonic development, embryos derived from parents heterozygous for the deficiency *Df(2L)PC4*, which removes *thr* (Figure 1.5), were examined by *in situ* hybridisation. During the syncytial divisions, all embryos exhibited uniformly high levels of transcript, as observed in the progeny of wild-type parents. During cellularisation and early germ band extension, a majority of the embryos again resembled wild-type. At these stages and later in development, however, approximately one quarter of the progeny exhibited dramatically lower levels of *thr* transcripts in all cells, except the pole cells (Figure 4.4I-K). These embryos presumably correspond to the homozygous deficiency embryos. Double staining with the DNA stain Hoechst 33258 showed that, as expected, the unstained fully germ band extended

embryos exhibited the mitotic defect characteristic of homozygous *thr* embryos (data not shown).

These findings indicate that maternal *thr* mRNA is rapidly degraded at the time of cellularisation and that *thr* transcripts observed late in cycle 14 and in subsequent cycles are the product of zygotic transcription. The transition from maternal transcripts to zygotic transcripts during cycle 14 is not, however, marked by a discernible stage in which *thr* transcripts are absent.

At higher magnification, the distribution of *thr* mRNA in pole cells was observed to be punctate and perinuclear (Figure 4.4J and results not shown). Such a subcellular distribution appears identical to that observed in pole cells for cyclin B mRNA (Lehner and O'Farrell, 1990b), which resides in cytoplasmic granules. The irregular, granular distribution of *thr* mRNA within germ line cells is also observed in somatic tissues (see staining in cells of PNS, Figure 4.4E), and contrasts with the uniform cytoplasmic staining observed for many other types of transcripts (for example see Richardson et al., 1993).

4.6 Discussion

The evidence presented here argues strongly that the early requirement for zygotic expression of *thr*, as revealed by the mutant phenotype, results from the instability of the maternal mRNA during cycle 14. The maternal *thr* mRNA appears to be destabilised upon cessation of the syncytial divisions. An apparently constant pool of maternal *thr* mRNA was found in all syncytial embryos. In embryos homozygous for a *thr* deficiency, this pool was observed to rapidly decrease following cessation of the syncytial divisions, so that at the time of cellularisation *thr* mRNA levels were reduced to undetectable levels in all cells except pole (germ line) cells. In normal embryos, however, the levels of *thr* mRNA were maintained at a much higher level during these stages, showing that zygotic transcription was supplementing the rapidly degrading maternal mRNA.

The disappearance of maternal *thr* transcripts at this time is consistent with the temporal onset of the mutant phenotype. Although it is possible that trace amounts of mRNA may persist after the precipitous decline in levels at the commencement of cycle 14, it is more likely that limited function in mutants is conferred by the persistence of

protein. In embryos homozygous for amorphic alleles, sufficient *thr* product is presumably translated from the maternal *thr* mRNA prior to its degradation for a normal 14th mitosis to occur, and perhaps for a rudimentary mitosis 15. Such a persistence of the maternally derived *thr* protein is also suggested by the observation that the maternal *thr* genotype affects the time at which the mutant phenotype becomes evident (D'Andrea et al., 1993).

The dependence of the zygotic phenotype on the maternal genotype, the absence of any stage-specific regulation of *thr* transcripts during cycles 14 and 15, and the correlation between expression and proliferation in embryonic and postembryonic development, all argue against the scenario that the *thr* embryonic phenotype results from a developmentally restricted requirement for *thr* function. Rather, these results are consistent with the idea that *thr* function is provided maternally and that in homozygous mutant embryos the maternal contribution becomes insufficient prior to mitosis 15. At this stage the mutant phenotype manifests itself because of a failure to produce functional zygotic *thr* products.

The apparent stability of the maternal *thr* mRNA during the syncytial divisions, compared with the instability during cellularisation, suggests that upon completion of the syncytial divisions, RNA degradative pathways, specific for certain transcripts, are activated. The change in stability is dramatically evident for maternal *string* transcripts (Edgar and O'Farrell, 1989) and for maternal *D. melanogaster* cyclin E transcripts (Richardson et al., 1993). The basis for the decreased stability of maternal transcripts, following the syncytial divisions, is yet to be determined for any of these genes.

If maternally conferred message is being actively degraded concomitant with zygotically initiated transcription, then some mechanism should exist to differentiate between transcripts of maternal and zygotic origin. Perhaps recognition sequences or structures are present in the mRNA 3' and 5' UTRs where polymorphisms have been detected. The maternal form may correspond to the -58±5 transcript, identified by primer extension analysis, present in abundance only in early embryos (Figure 4.3).

Experiments designed to further elucidate the function of the *thr* product in mitosis are described in the next chapter.

Chapter 5 (Metaphase): Immunodetection

5.1 Background

Evidence presented in Chapter 3 established that *three rows* encodes a "pioneer" protein essential for mitotic chromosome disjunction. However its precise role in this process is unknown. With few clues revealed by the sequence encoded by the ORF, or the pattern of gene expression, other stategies were required to elucidate its precise function. A powerful technique in this regard is the utilisation of antibodies specifically directed to the encoded product.

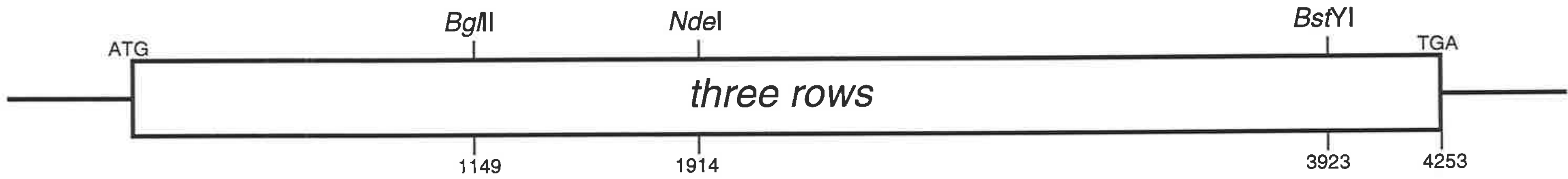
The aim of this section of work was firstly to obtain substantial quantities of three rows derived protein by bacterial expression. This protein would be employed to generate specific antibodies by immunisation and the three rows directed antibodies purified from the immune sera. Application of antibodies to whole mounts of embryos should reveal the subcellular location of the antigen. In particular, localisation of three rows to a particular element of the mitotic apparatus would enable informed speculation as to function, whether it be signalling, structural or mechanical.

5.2 Antibody production

Two bacterial expression systems were exploited to raise antibodies specific to three rows (Figure 5.1). The C-terminal 1014 aa of the derived product (Figure 5.1) were expressed as a 142 kDa glutathione-S-transferase (GST) fusion protein (Figure 5.2A) using the pGEX vectors (Smith and Johnson, 1988). A nonchimaeric 77 kDa protein (Figure 5.2A) consisting of 670 aa of the *thr* putative product (Figure 5.1) was expressed with the T7 transcription/translation system (Studier and Moffatt, 1986).

Although the pGEX system permits the purification of the fusion protein based on the affinity of GST for glutathione agarose, this could not be applied for the GST-three rows fusion. Following induction of expression and bacterial lysis the fusion protein was recovered almost exclusively in the pellet. The small amount of soluble

Figure 5.1 Bacterial expression constructs employed to produce three rows derived proteins, in relation to the *three rows* ORF.

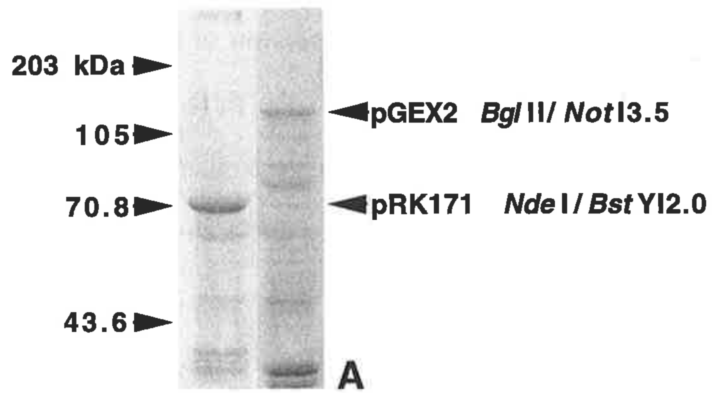


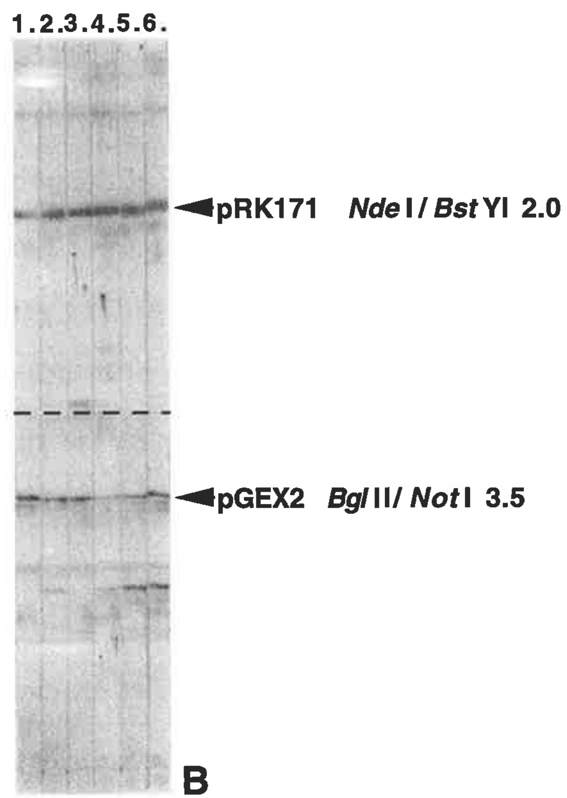
pGEX2 *Bgl*II/*Not*I 3.5

pRK171 *Nde*I/*Bst*YI 2.0

Figure 5.2 Three rows derived bacterial expression products and detection of immune response in rabbits inoculated with them.

A. Coomassie stained SDS-PAGE of three rows derived bacterial expression products. Mobility of protein molecular weight markers is arrowed. B. Detection, by western blot analysis, of immune response in rabbits inoculated with recombinant protein Nitrocellulose strips consist of two separate gels. Rabbits: 1-3 inoculated with pGEX2 *Bgl*II/*Not*I 3.5, 4-6 with pRK171 *Nde*I/*Bst*YI 2.0. Position of bacterial expression products is arrowed. All rabbits produce immune sera that reacts with both bacterial expression products.


203 kDa
105
70.8
43.6
pGEX2 BglII/ NotI3.5
pRK171 NdeI/ BstYI2.0
A


1.2.3.4.5.6.
pRK171 NdeI/ BstYI 2.0
pGEX2 BglII/ NotI 3.5
B

protein was capable of binding to glutathione agarose, but could not be eluted. Consequently, for antibody production, the recombinant protein was resolved on an acrylamide gel and a homogenised gel slice containing the protein was used for immunisation. The same technique was employed for the T7 expressed protein.

The rabbits immunised with the bacterially expressed, three rows derived proteins exhibited an immune response as judged by the reaction of their sera to the recombinant proteins on western blots (Figure 5.2B). However the failure of the crude immune sera to reproducibly detect a *D. melanogaster* product on westerns or in embryo whole mounts made it necessary to purify the three rows specific antibodies.

5.3 Antibody purification

Three rows specific antibodies generated in rabbits inoculated with recombinant protein were purified on the basis of their affinity for the T7 expressed protein. This was also feasible for immune sera against the GST fusion protein because of the considerable stretch of sequence shared between the two bacterially expressed proteins (Figure 5.1). Such an approach also had the desirable outcome of excluding antibodies directed against the GST portion of the fusion protein. The T7 expressed protein was enriched by resolution on SDS-PAGE, electroeluted from an excised gel slice (Figure 5.3A) and covalently coupled to Affigel 10 to form the matrix of an affinity column.

Antibodies with both low and high affinity for the T7 expressed protein were purified with the affinity column as described (Kellogg and Alberts, 1992). Crude immune serum was passed over the column and, following extensive washing, antibodies that recognise the T7 protein with low affinity were eluted with 50mM HEPES pH 7.6, 1.4 M $MgCl_2$, 10% glycerol. High affinity antibodies retained on the column were eluted with 0.5% acetic acid, 0.15 M NaCl, and neutralised. Peak fractions were detected by their reactivity to recombinant protein on western blots (Figure 5.3B), pooled and concentrated. These high affinity antibodies are particularly suitable for western blotting and immunostaining (Kellogg and Alberts, 1992).

5.4 Western analysis

Whilst the antibodies purified with the affinity column from various immune sera clearly reacted to both bacterially expressed three rows derived proteins (Figure 5.3B), it was imperative to demonstrate that they recognised the *in vivo* product. This was accomplished by the detection in homozygous thr^{BH} embryos of a truncated product relative to that in wild-type (Figure 5.4). The molecular weight estimated from the mobility relative to protein standards was 150 kDa for the wild-type protein and 125 kDa for the mutant, which is in reasonably good agreement with 157 and approximately 132 kDa respectively predicted from the nucleotide sequence data (Chapter 3). Homozygous mutant embryos were selected based on their exhibition of the mitotic defect, which of necessity, was late in embryogenesis. The relatively low signal intensity relative to background is consistent with the low levels of protein detected in immunostainings of late embryo whole mounts (Section 5.5). Despite variation of conditions ECL detection gave unsatisfactorily high levels of background. However much of this appears to be a nonspecific product of the western blot, as it is also detected in the protein size marker gel track (Figure 5.4).

5.5 Immunolocalisation

Application of immunoaffinity purified antibodies to formaldehyde fixed embryos, and detection with HRP in combination with Hoechst 33258 staining of DNA, revealed a cell cycle dependent pattern of antigen localisation (Figures 5.5, 5.6). This was particularly marked in syncytial embryos where the metachronous nature of the cortical divisions made it possible to observe, in a single embryo, the transition from one mitotic stage to another. Occasionally embryos arose where the mitotic wave propagated from only one pole and, as a consequence, it was sometimes possible to observe the progress of almost an entire mitotic cycle across the long axis of the embryo (Figure 5.5 A, B).

The antigen is not detected in interphase (Figure 5.5 A, C, F and results not shown). However as chromosome condensation becomes apparent in prophase, the nucleus becomes progressively and uniformly stained except for some concentration coincident with chromatin (see arrows in inset, Figure 5.5C). As the nuclear envelope

Figure 5.3 Purification of pRK171 *Nde*I/*Bst*YI 2.0 bacterially expressed protein, and utilisation for purification of three rows specific antibodies by immunoaffinity chromatography.

A. Coomassie stained SDS-PAGE of pRK171 *Nde*I/*Bst*YI 2.0 bacterial protein prior to, and following purification by electroelution. B. Detection, by western blot analysis, of three rows specific antibodies, from rabbit immune serum 3, eluted from a pRK171 *Nde*I/*Bst*YI 2.0 bacterial protein column. Nitrocellulose strips consist of two separate gels.

Unpurified | Gel purified
A

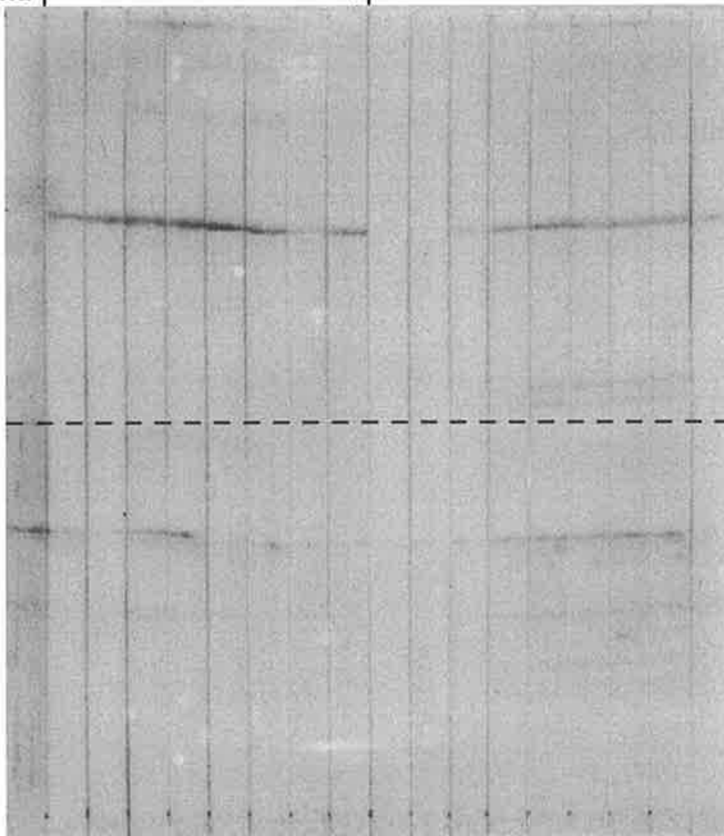

Unbound
Low affinity eluate:
1. 2. 3. 4. 5. 6. 7. 8.
High affinity eluate:
1. 2. 3. 4. 6. 8. 10.12.14.
pRK171 *Nde* I / *Bst* YI 2.0
pGEX2 *Bgl* II / *Not* I 3.5
B

Figure 5.4 Detection of three rows in wild type and *thr*^{BH} embryos by western blot analysis.

1. Protein molecular weight markers.
2. *thr*$^{BH/BH}$.
3. *thr*$^{BH/+}$.
4. *thr*$^{+/+}$.

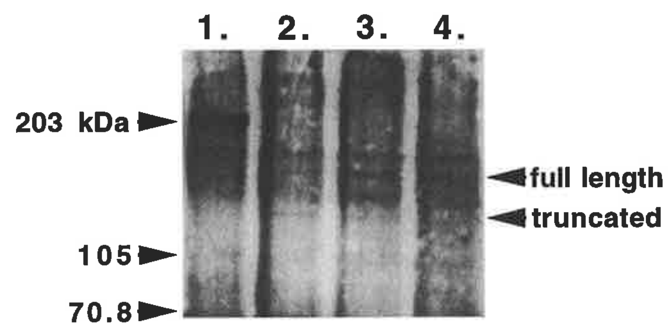

1.
2.
3.
4.
203 kDa
105
70.8
full length
truncated

Figure 5.5 Immunostaining of syncytial blastoderm embryo whole mounts with anti-three rows antibodies.

Antigen was detected with HRP and the DNA counterstained with Hoechst 33258.

A. Whole embryo exhibiting, from left to right, nuclei in prophase through to interphase.

B. Whole embryo, metaphase to telophase.

C. Interphase-metaphase, inset: early prophase nucleus showing staining associated with condensing chromatin (arrows).

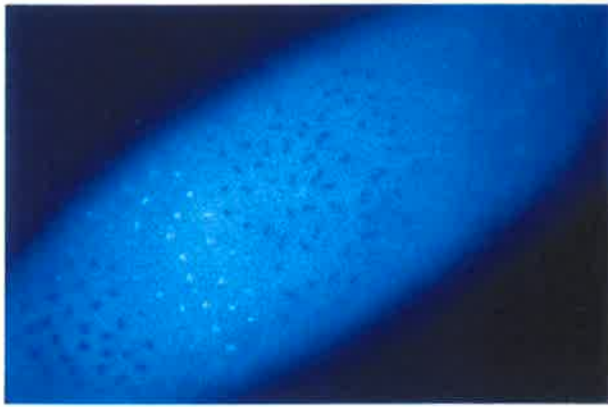
D. Prophase-anaphase.

E. Metaphase-anaphase.

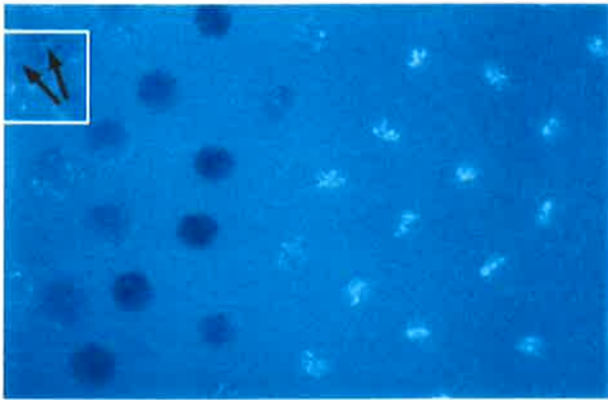
F. Telophase-interphase.

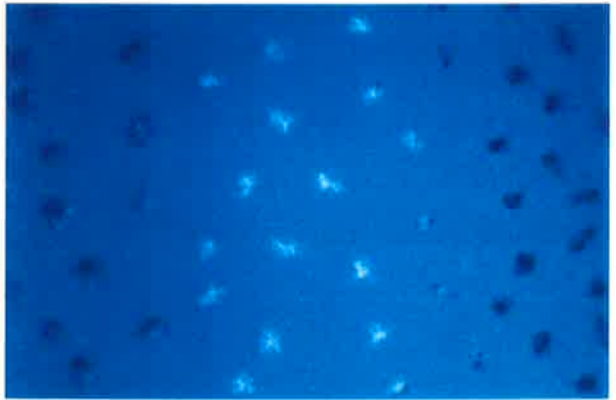
G. Whole embryo showing staining in pole cells (bottom right).

G'. Internal focal view of same embryo as in "G." showing vitellophage nuclei staining.

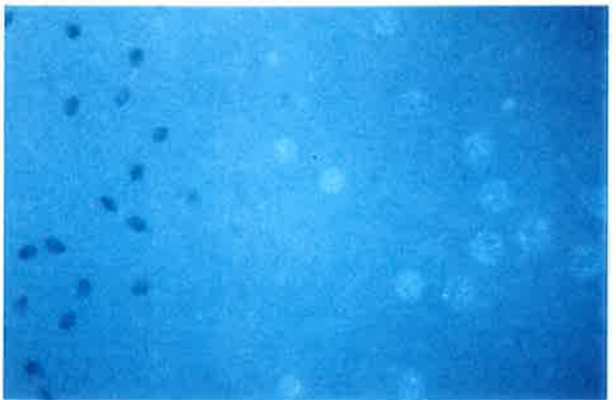

A

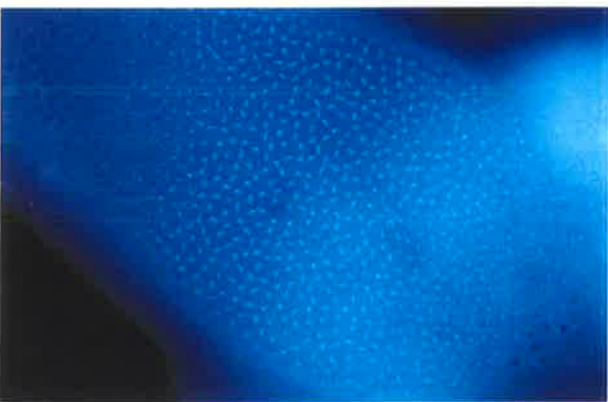
B


C


D

E


F


G

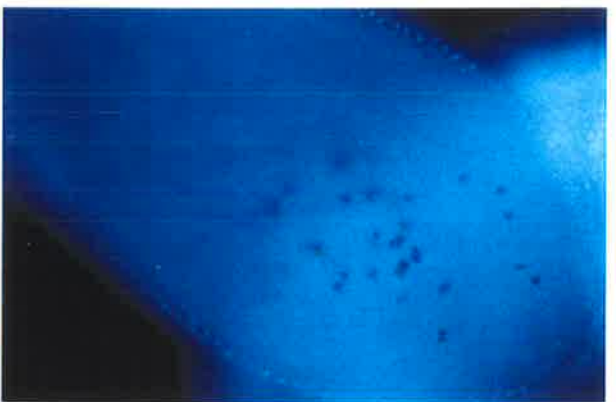

G'

Figure 5.6 Immunostaining of cellularised embryo whole mounts with anti-three rows antibodies.

Antigen was detected with HRP and in some cases the DNA visualised by counterstaining with Hoechst 33258. All are dorsal views of germ band extended embryos unless otherwise stated.

A. Staining of presumptive subepidermal neuroblasts in the neurogenic region.

B. Lateral view showing germ band staining and persistent staining of irregularly dispersed presumptive vitellophage nuclei.

C. Anterior end with fluorescent visualisation of DNA, showing staining in presumptive neuroblasts (arrows) at the margins of the brain lobes.

D. Lateral view of germ band retracted embryo showing general lack of staining, except for pole cells associated with the posterior midgut.

E. High magnification fluorescent visualisation of epidermal cells showing staining in cells in early anaphase (eA), anaphase (A), and telophase (T).

E'. Visible light view of same embryo as in "E".

F. Fluorescent visualisation of DNA in homozygous *Df2L)Pcl7B* (left) and phenotypically wild type (right) embryos.

F'. Higher magnification visible light views of same embryos as in "F" showing absence of staining in the deficiency embryo.

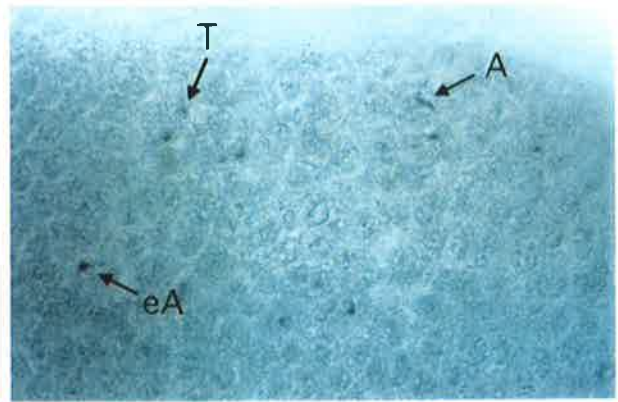
A

B

C

D

E



E'

F

F'

undergoes partial breakdown and chromosomes congress in prometaphase the nuclear staining is lost, until it is essentially undetectable at metaphase (Figure 5.5 B, C, D, E). Coincident with the first manifestation of chromosome movement in anaphase, the antigen again becomes detectable on the chromosomes, first appearing at the regions where polewards movement commences (Figure 5.5 D, E). These are presumably regions around the kinetochore. As anaphase proceeds, the staining localises to the entire chromosome and becomes progressively stronger (Figure 5.5 D, E), appearing to reach maximum intensity as the nuclear envelope reforms around the chromosomes in telophase (Figure 5.5F). The staining then declines as the nuclei take on the enlarged appearance characteristic of S phase (Figure 5.5F).

Intense staining of yolk nuclei is also observed in many syncytial embryos (Figure 5.5G'). Staining of vitellophage nuclei persists in development and is sometimes detected in gastrulating embryos (Figure 5.6B).

Consistent with the elevated levels of mRNA detected in them (Section 4.5), pole cells are often strongly immunostained, relative to the neighbouring syncytium/cells (Figure 5.5G). This staining is persistent, and can be detected late in embryogenesis when the pole cells are internalised and associated with the posterior midgut (Figure 5.6D).

The pattern of antigen localisation in embryos post cycle 13, although more difficult to observe because of lower signal intensity and smaller nuclei, appears consistent with that in syncytial embryos. Early in germ band extension, the nuclei of cells stain in a manner that reflects, at least in part, the pattern of mitoses (Figure 5.6 A, B). In particular, a characteristic arrangement of three subepidermal rows of large cells on either side of the ventral midline (Figure 5.6A) is consistent with the pattern of CNS neuroblast distribution at this stage. Because of the internalisation of these cells it is difficult to correlate this staining with a particular mitotic stage.

In epidermal cells the antigen exhibits a cell cycle dependent pattern of detectability, similar to the precellular divisions, being most evident in anaphase and telophase when associated with the chromosomes (Figure 5.6 E, E'). However the prophase accumulation, observed in syncytial embryos, is not marked during cellular

divisions. Staining declines in level as embryogenesis proceeds until undetectable, in all tissues of germ band retracted embryos, except isolated cells in the proliferating margins of the brain lobes (Figure 5.6D).

The specificity of immunostaining was demonstrated by the the loss of antigen in homozygous *Df(2L)Pcl7B* embryos exhibiting the *thr* phenotype late in embryogenesis (Figure 5.6 F, F').

Immunofluorescent staining of embryos confirmed the cell cycle dependent pattern of antigen localisation observed for HRP detection (Figure 5.7). Confocal and epifluorescent imaging of syncytial embryos revealed staining of nuclei in prophase, absence in metaphase, and mobilisation to chromosomes in anaphase and telophase(Figure 5.7 A, B, B', C). DNA associated staining of chromosomes during anaphase and telophase was also observed in cellularised embryos (Figure 5.7 E, E').

Interpretation of the immunolocalisation pattern was hampered by the variable degree to which centrosomes are also stained, particularly in methanol fixed embryos, in what appears to be a nonspecific fashion (see frontpiece). In homozygous *Df(2L)Pcl7B* embryos, in which nuclear/chromosomal staining is abolished in cycles 15 and 16, centrosomal staining can be detected fluorescently (Figure 5.7 D, D' and results not shown). The centrosome is a structure particularly prone to artefactual immunostaining (J. Raff, pers. comm.). This apparently artefactual staining makes it impossible to rule out a *bona fide* association of three rows with the centrosome. As a negative control, embryos were incubated with the IgG fraction isolated from preimmune sera. Even when preimmune IgGs were applied at 500 times the concentration of the affinity purified antibodies, no staining was detected (data not shown).

5.6 Discussion

The considerable effort expended to obtain specific antibodies capable of *in situ* detection is evidence that three rows may not be a particularly immunogenic protein. Satisfactory preparations of affinity purified polyclonal antibodies were not obtained from the sera of subcutaneously immunised mice and rats, despite clear ellicitation of an immune response. Mice were also inoculated intraperitoneally with approximately 1 mg of

Figure 5.7 Immunofluorescent staining of embryo whole mounts with anti-three rows antibodies.

Embryos have been double stained with a Texas-Red conjugated secondary antibody to recognise three rows (red), and chromomycin A_3 for DNA (green), and visualised by epifluorescent (B, B', D, D', E, and E') or confocal (A and C) microscopy. All are wild type syncytial embryos unless otherwise stated.

A. Double imaging of DNA and three rows in embryo in stages, from left to right, of prophase through to anaphase.

B. Visualisation of DNA in nuclei in anaphase-telophase.

B'. Same embryo as in "B" showing visualisation of three rows.

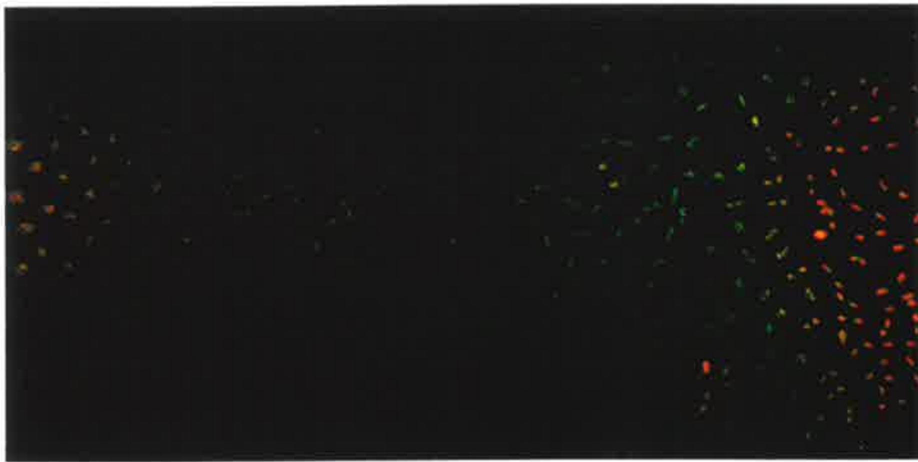
C. Double imaging of DNA and three rows in nuclei in prophase-metaphase.

D. Visualisation of DNA in cells of homozygous germ band extended *Df(2L)Pcl7B* embryo.

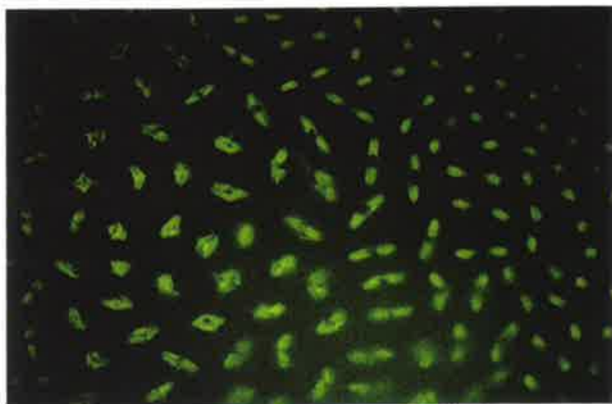
D'. Same embryo as in "D" showing persistent nonspecific staining by anti-three rows antibodies of presumptive centrosomes.

E. Visualisation of DNA in epidermal cells of germ band extended embryo.

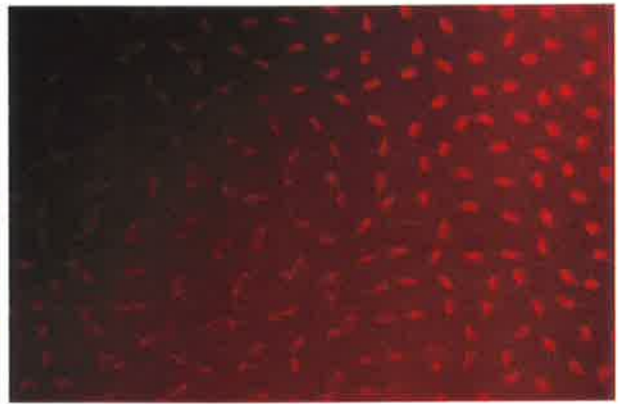
E'. Same embryo as in "D" showing DNA associated three rows staining in anaphase and telophase (bottom right).



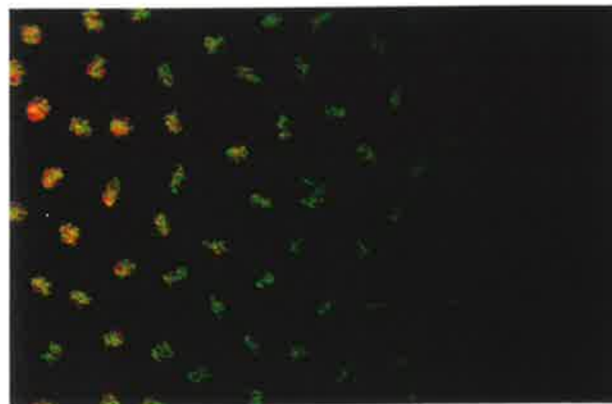
A



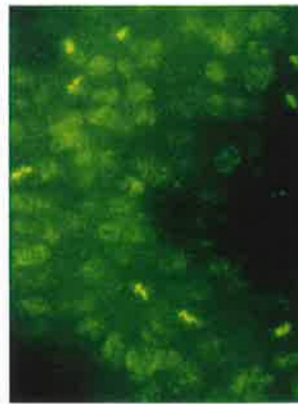
B



B'

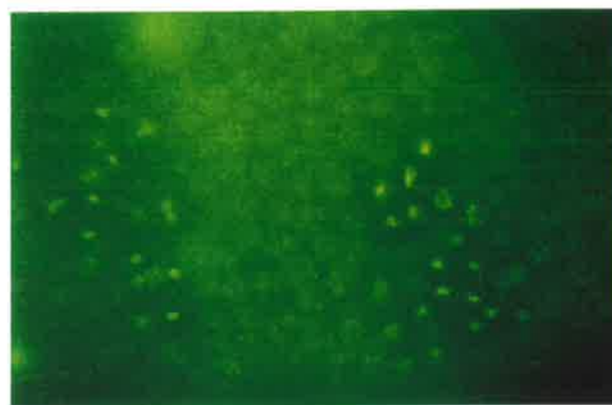


C



D

D'



E

E'

recombinant protein, along with Freund's complete adjuvant, for the purpose of obtaining monoclonal antibodies. Of 35 hybridoma cell lines isolated from mice immunised with the T7 expressed protein (J. Wrin, pers. comm.), none had more than a weak affinity for recombinant protein on westerns (data not shown). It is possible that the isolation of efficacious antibodies from rabbits is a product of the large volumes of sera obtained, implying that specific high affinity antibodies may represent a very small proportion of total circulating levels. It is also possible that because of masking by other proteins or posttranslational modifications *in vivo*, or because of major differences in conformation between the the bacterially expressed/SDS-PAGE resolved, and the *in vivo* expressed forms, that antibodies to very few native epitopes were present in the immune sera raised.

The dynamic pattern of three rows localisation, whilst unique, is similar to some other antigens associated with the mitotic spindle. Several antigens in *D. melanogaster* also localise to the chromosomes in anaphase. The zw10 protein, which is excluded from the nucleus in telophase and interphase, accumulates within the spindle envelope in prometaphase, appears spindle associated in metaphase, and rapidly localises to a region at or near the kinetochores in anaphase (Williams et al., 1992). Prior to cycle 10 in syncytial embryos proliferating cell nuclear antigen (PCNA) stains chromosomes in anaphase, but not metaphase (Yamaguchi et al., 1991). In subsequent cycles PCNA becomes associated with chromosomes/nuclei at progressively later stages of the cell cycle, which is consistent with its function in DNA replication.

There exists in *D. melanogaster* a protein with a cell cycle dependent pattern of localisation reminiscent to that of three rows. An antigen recognised by "Aj1", one of a collection of monoclonal antibodies directed against nuclear proteins (Frasch et al., 1986) (Section 1.5.5), occupies the nucleus of syncytial embryos in prophase then disperses to surround the spindle in metaphase whilst being excluded from the chromosomes. In anaphase it specifically associates with the chromosomes, remains present in telophase, and appears also to persist throughout interphase. Following cellularisation the pattern of Aj1 staining in mitosis is similar to that in syncytial embryos except that in interphase the antigen localises not to the nucleus, but the nucleolus. In spite of the similarities to three rows, Aj1 clearly recognises a distinct protein of 47 kDa on westerns (Frasch et al.,

1986). There are other differences which may be significant, such as the continuing detection of the antigen around the spindle in metaphase, and its persistence during interphase, particularly its nucleolar localisation following cellularisation. In the latter case, it may be pertinent to note that the organelles which stain with Aj1 (the nucleolus), and (nonspecifically) with anti-three rows antibodies (the centrosome) are both singular, punctate and nuclear membrane associated during interphase.

Are the abrupt transitions in the detection of three rows in syncytial embryos the product of cycles of protein synthesis and degradation, or the result of redistribution of a relatively constant pool of protein? The velocity of the "mitotic wave" during the metachronous syncytial divisions makes it unlikely fluctuations in antigen detection are caused by proteolysis and *de novo* translation. It has been estimated that it takes fewer than 30 seconds for the wave to propagate from the pole to the equator, although intervals of up to 2.5 min have been observed (Foe and Alberts, 1983). As staining can be observed in nuclei that are only one intervening nucleus away from nuclei with no staining (Figure 5.5 C, D, E), spanning less than 10% of the length of an embryo, it would appear that the translocations of protein in both prometaphase and anaphase may take less than 5 seconds and probably no more than 30 seconds. Such intervals are difficult to reconcile with protein degradation and synthesis. Periods of 10-12 s have been estimated for the *D. melanogaster* zw10 protein, which has a similarly rapid pattern of localisation to the chromosomes in anaphase (Williams et al., 1992).

If protein is being transported from the nucleus in prometaphase, why is it not detected in the cytoplasm in metaphase? It is conceivable that protein dispersed into the cytoplasm may be below the threshold of immunodetection, although the apparent levels in prophase and anaphase make this unlikely. Alternatively, the antigen may be immunologically masked in metaphase, as a consequence of association with other proteins in a complex, due to posttranslational modification, or for some other reason.

Although the pattern of immunolocalisation is not particularly redolent of a microtubule associated protein (MAP), experiments were performed to ascertain if three rows had an affinity for microtubules. Western analysis of proteins isolated by cosedimentation with taxol stabilised microtubules (Kellogg et al., 1989) demonstrated a

clear enrichment for DMAP 190 (antibody supplied by J. Raff and D. Kellogg), but failed to detect three rows (data not shown).

The distinctive pattern of three rows localisation revealed by immunostaining is consistent with some of the previously observed (Section 3.5) characteristics of the derived product. The apparent association with chromosomes is consistent with the basic nature of the protein. It is possible three rows interacts directly with DNA as do other basic proteins like histones, although it is also feasible the association occurs via interactions with other proteins. Although probably not degraded in prometaphase, the identified PEST sequences may target the protein for degradation in interphase. The accumulation of protein in the nucleus during prophase correlates with the presence of nuclear targeting sequences. Furthermore, the cell cycle dependent fluctuations in nuclear localisation may be a product of phosphorylation changes at some of the protein kinase recognition sites that are coincident with, and possibly modulate the activity of, the best candidate nuclear targeting sequence (Table 3.2).

The pattern of three rows immunolocalisation is also consistent with the *three rows* mutant phenotype. Essentially, chromosome disjunction may be defective in mutants because the protein fails to migrate to the chromosomes at the initiation of anaphase, or because the protein migrates but is nonfunctional. Translocation of the protein could be a consequence of MPF activation or other associated changes at the metaphase-anaphase transition which would explain why chromosome disjunction is defective in mutants whilst cell cycle progression is unimpeded.

With the lack of sequence similarity of the derived product offering no hint, the pattern of immunolocalisation is presently the best indicator of three rows function. Even though the mutant phenotype implies three rows is only essential for anaphase chromosome movement any suggestions for function must be able to account for its detection, in syncytial embryos, in prophase as well as anaphase. One explanation is that three rows *does* function in prophase but that, either, phenotypic manifestation in mutants has not been elucidated by our superficial examination, or the consequences of the prophase defect do not become apparent until chromosomes have congressed. Another possibility is that three rows is not required early in mitosis, but that its accumulation in

the nucleus in prophase is a means of sequestering sufficient quantities of protein in the vicinity of the presumptive spindle, particularly during the extremely rapid syncytial divisions, to mediate its essential function late in mitosis. Accordingly, the protein detected in prophase may not be functional, requiring some anaphase specific form of post-translational modification to become activated or localised.

If three rows is required both early and late in mitosis there are at least two possible functions. One is suggested by the antigen's association with chromosomes in prophase, and anaphase, stages marked by states of intermediate chromosome condensation. Chromosome condensation, however, appears to be unaffected in mutants (D'Andrea et al., 1993) and there are no precedents for defective chromosome condensation inhibiting disjunction. Detection of the antigen in both prophase and anaphase is also evidence for a general requirement in chromosome movement. However the mutant phenotype argues against a role in metaphase alignment, and immunostaining appears to show that the antigen is dispersed in prometaphase before chromosomes have fully congressed (Figures 5.5 C, D, 5.7C).

If three rows function is confined to anaphase then it could function either in a signalling, or structural capacity to engender chromosome movement. Three rows could be a kind of "licensing factor" whose rapid translocation to the chromosomes at the initiation of anaphase in some way signals them to move polewards. Alternatively, it could have a more direct role in chromosome movement by altering the relatively static interaction between the kinetochore and the spindle MTs that exists at metaphase. Otherwise it may be responsible for dissolution of the bonds that join sister chromatids until the commencement of disjunction. All of these roles may only require redistribution of protein to the kinetochore/centromere region. Accordingly, the mass decoration of the chromosomes in syncytial anaphases could be a product of the high levels of protein at this developmental stage, or to ensure the equitable distribution of the protein to the daughter nuclei.

The role of three rows in mitosis later in development, implied by the data on gene expression (Section 4.3), remains to be immunologically investigated. Results from a preliminary immunostaining of third instar larval brain lobes reveal localisation to giant

neuroblast cells (data not shown) which are known to be proliferating at this stage. It will be of interest to extend this investigation to look for the presence of three rows in imaginal and gametogenic (both mitotic and meiotic) proliferation.

Further insights into *three rows* function by the characterisation of a diverged homologue are described in the next chapter.

Chapter 6 (Anaphase): Isolation of a homologue from *D. erecta*

6.1 Background

In circumstances where a gene has been shown to encode a "pioneer" protein of undetermined function, the isolation and characterisation of the homologous gene from an appropriately diverged species has the potential to yield functional insights. The rationale is that domains that have conserved their sequence during the course of evolution do so because of functional constraints on that sequence. Accordingly, in a reductionist approach, these conserved regions would be targeted for detailed investigation of function. For example, the cyclins exhibit considerable evolutionary divergence in their amino acid sequence such that from yeast to man only a single conserved region can be detected. This domain, the "cyclin box" is absolutely required, being responsible for the cyclin's interaction with, and activation of, the CDK catalytic subunit.

In a manner that approximates their degree of sequence conservation, homologues of *D. melanogaster* genes are usually isolated from one of three different "sister" groups. At the extreme end of the conservation scale are genes whose homologues have been isolated from yeast or man. The histones are an example of genes which have been conserved (Wells and McBride, 1989) across the estimated 1-2 billion years of eukaryote evolution. The homologues of intermediately conserved *D. melanogaster* genes are often detected in other arthropods, like crustaceans, or in representative species of other insect orders such as *Tribolium*, *Manduca* or *Schistocerca*. Genes that have been conserved during this approximate 500 my period of divergence include the pair-rule gene *fushi-tarazu* (for example see Dawes et al., 1994). Looking closer afield, the characterisation of homologues of less evolutionarily conserved genes have exploited the 60-80 million year (Beverley and Wilson, 1984; Spicer, 1988) radiation of the genus *Drosophila*. At least 11 homologues of *D. melanogaster* genes having been isolated from *D. virilis* (reviewed in O'Neil and Belote, 1992), the species of choice for this work.

It might reasonably be predicted that a product like three rows, which has been shown by mutation to perform an essential role in a universal process such as mitosis, would be highly conserved. The aim of this work was first to define, by Southern hybridisation, the extent of conservation of the *three rows* coding sequences. This information would be applied to isolate a homologue from a species at the limit of detectability with the heterologous probe. Comparison of the derived sequence of the homologous product with that of *D. melanogaster* should identify evolutionarily conserved domains, that could be further analysed.

6.2 Genus blot

As a starting point for the isolation of a diverged *thr* homologue, the extent of conservation of hybridising sequences in the genus *Drosophila* was gauged using a genomic Southern "genus blot". On the basis of schemes for the phylogeny of *Drosophila* (Beverley and Wilson, 1984; Lachaise et al., 1988) (Figure 6.1), nine species were chosen, representing a range of suggested divergence times from the *D. melanogaster* line. Along with *D. melanogaster*, their genomic DNA was isolated, restricted with *Hin*dIII, resolved by electrophoresis and membrane immobilised. The genus blot was probed with the UJA8 cDNA clone under nonstringent hybridisation and washing conditions as suggested (O'Neil and Belote, 1992).

Remarkably, it was not possible to detect hybridising sequences in species thought to have shared an ancestor with *D. melanogaster* more than 20 mya (Figure 6.1). Under the least stringent of conditions sequences complementary to *thr* could only be detected reproducibly in the genomes of species up to and including *D. eugracillus*. A major radiation is believed to have occurred about 60 mya leading to the divergence of the subgenus *Drosophila*, that includes *D. virilis*, from the subgenus represented by *D. melanogaster*, the *Sophophora* (Beverley and Wilson, 1984; Spicer, 1988). Species from the subgenus *Drosophila* contain no *thr* related sequences that can be detected by low stringency genomic Southern hybridisation (Figure 6.1).

The presence and integrity of the genomic DNA on this genus blot has been confirmed. Stripping and reprobing of the membrane allowed the detection in the subgenus

Drosophila, of sequences complementary to the *D. melanogaster* genes *Polycomblike* and *deadringer* under relatively stringent conditions (washing with 0.1 x SSC at 42°C, S. Robert and D. Kortschak, pers. comm.).

6.3 Isolation of homologous sequences

In spite of the results of the genus blot, efforts were made to isolate, by library screening, sequences homologous to *thr* from *D. virilis*. This was based on the greater sensitivity expected for hybridisation to a library filter of nonconfluent, high titre plaques. Attempts were made to screen a number of platings of three independently constructed *D. virilis* genomic or cDNA libraries under suggested nonstringent conditions (O'Neil and Belote, 1992). A number of weakly hybridising clones were isolated and the hybridising sequences therein subcloned. Extensive sequencing and analysis of the nucleotide sequences and those of the notional amino acids encoded revealed no significant similarities to *thr* (data not shown).

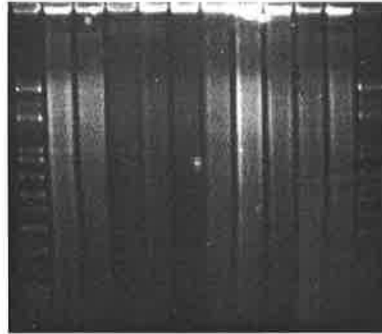
As a consequence of these results it was decided to isolate a *thr* homologue from a species more closely related to *D. melanogaster*, one in which *thr* related sequences could be reproducibly detected on the genus blot (Figure 6.1). A number of aliquots were obtained of a genomic library (Hickey et al., 1991) from *D. erecta*, a species believed to have diverged from the *D. melanogaster* line about 17 mya (Figure 6.1). One aliquot of this library (obtained from A. Lohe and D. Hartl, Washington University, St Louis, MO) yielded 7 hybridising clones out of approximately 5 x 10^5 plaques screened with cDNA UJA8. Restriction analysis of the clones demonstrated they were related, and of two types (data not shown), suggesting a low level of complexity in the library aliquot, probably a result of the aliquot having been amplified at least twice. Southern hybridisation indicated that sequences related to UJA8 were confined, in the most extensive of the two insert types, to two *Eco*RI fragments of 1.0 and 3.2 kb (data not shown). These were subcloned for sequence determination (Section 6.4).

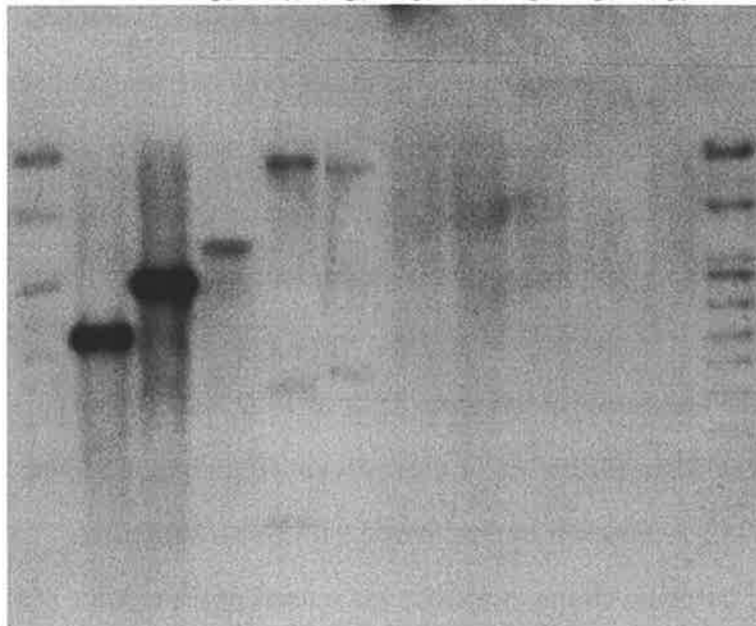
Although *D. melanogaster* and *D. erecta* are each equally diverged from *D. virilis* (Figure 6.1) it is possible that the *D. erecta thr* may be a better probe for isolating *thr* of

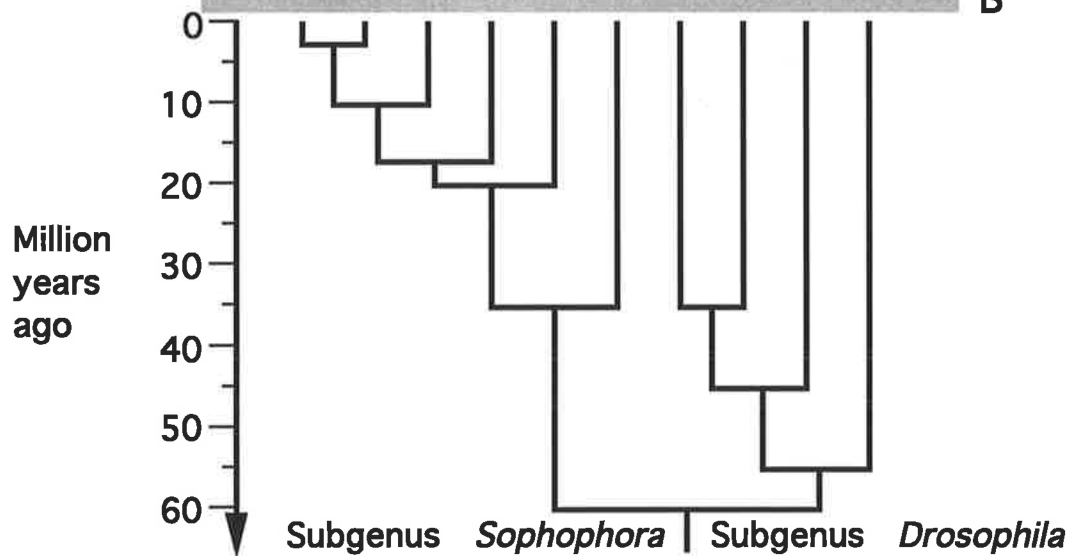
Figure 6.1 Genus blot and suggested phylogeny of *Drosophila*.

A. Ethidium bromide stained gel. B. Southern blot. Blot was hybridised with UJA8 cDNA and washed under nonstringent conditions. A suggested phylogeny for the genus *Drosophila* and timescale (see text for references) is aligned with corresponding gel tracks on the Southern blot. Hybridising sequences can only be detected reproducibly in genomes of species up to and including *D. eugracilus*. Gel tracks:

1. *D. melanogaster*
2. *D. simulans*
3. *D. yakuba*
4. *D. erecta*
5. *D. eugracilis*
6. *D. ananassae*
7. *D. virilis*
8. *D. robusta*
9. *D. hydei*
10. *D. funebris.*


1. 2. 3. 4. 5. 6. 7. 8. 9.10.
A


1. 2. 3. 4. 5. 6. 7. 8. 9. 10.
B


0
10
20
30
40
50
60
Million years ago
Subgenus *Sophophora*
Subgenus *Drosophila*

D. virilis. However an attempt to screen a *D. virilis* library with *D. erecta thr* sequences as a probe was similarly unsuccessful (data not shown).

6.4 Characterisation of *D. erecta three rows*

Sequence of most of the *three rows* genomic region from *D. erecta* has been obtained, from 170 nt 3' of the initiation codon identified in *D. melanogaster* to over 160 nt 3' of the termination codon (Figure 6.2). This precludes comparison of the 57 N-terminal amino acid residues of *D. melanogaster* three rows with the corresponding sequences in *D. erecta*.

The *D. erecta thr* ORF appears to be interupted by two small introns, at identical position to those in *D. melanogaster*. No direct evidence has yet been obtained that these regions are introns in *D. erecta*. However the occurrence of related sequences in these positions (Figure 6.3 A, B), the conservation of presumptive splice donator and acceptor sites (Figure 6.2), and the presence of in frame stop codons within the putative introns (Figure 6.2) all support the proposition that these regions are noncoding in *D. erecta*.

The ORF terminates at the same TGA as in *D. melanogaster* and strikingly similar sequences extend for a further 58 nt downstream (Figure 6.3C). The nucleotide sequence in the ORF is 88.3% identical to that shared with *D. melanogaster* (Table 6.1) although the 3' most 1 kb of common coding sequence has a level of divergence (85.6%) comparable to that of intron and 3' noncoding sequences (Table 6.1).

Table 6.1 Percentage identity* between genes of *D. melanogaster* and their homologues in *D. erecta*.

		three rows	*transformer*[1]	α-amylase[2]	*Sgs-3*[3]	*Adh*[4]	*ref(2)P*[5]
UTR:	- 5' plus 3'			85.0			
	- 5'	-	85.5	-	75.6#		
	- Intron	81.1, 86.0+	86.0, 67.8+	n.a.	69.3	61.0	n.a.
	- 3'	84.8	93.7	-	68.5#		
ORF:		88.3$	87.7	86.45	84.8	95.7	90.6
Protein sequence		88.3$	77.3	95.75	84.6	96.1	86.3

* Percentage identity of pairwise compared residues, i.e. *relative insertions and deletions not scored as mismatches.*
n.a. not applicable.
\# Includes long stretches of nontranscribed sequence.
\+ *three rows* and *transformer* have two introns.
$ *D. erecta* sequence not complete at 5'/N-terminal end.
1,3,5 Encoded proteins contain repeated sequences of low complexity.

1 (O'Neil and Belote, 1992)
2 (Hickey et al., 1991)
3 (Martin et al., 1988) *scores deletions as mismatches.*
4 (Martin et al., 1988) *scores deletions as mismatches*, derived from data of (Bodmer and Ashburner, 1984) on *D. orena*, a close relative of *D. erecta.*
5 (Dru et al., 1993)

The (incomplete) derived product of the *thr* gene of *D. erecta* is a 1319 aa protein which, consistent with the nucleotide data, is 89.7% identical to that of *D. melanogaster* over the N-terminal 989 amino acids of shared sequence, and 84.2% over the C-terminal 333 (Figure 6.4). The two polypeptide sequences are colinear over the common region except for the relative deletion, in *D. erecta*, of three amino acids near the C-terminal end (Figure 6.4). Amino acid substitutions relative to three rows of *D. melanogaster* are mostly singular and appear randomly dispersed throughout the product except for the slightly elevated levels at the C-terminus. There are no strikingly conserved domains embedded in regions of diverged sequence. Only one of the consensus amino acid

Figure 6.2 Nucleotide and derived amino acid sequence of *three rows* from *D. erecta.*

The nucleotide sequence of the *D. erecta* gene has a long open reading frame encoding an incomplete theoretical protein of 1319 amino acids. Coding sequence is shown in upper case, and putative intron and 3' noncoding sequence are shown in lower case. *Eco*RI sites are underlined. Consensus splice donator and acceptor sites, and in frame termination codons in the putative introns are shown in bold. The derived amino acid sequence is shown in one letter code below the nucleotide sequence.

```
     EcoRI
   1 GAATTCGGATCTTTACTGCGACATTATAGGCATAATGTTGCCACGTGTAGTTCCCTTTGAGGAGAAGCCCAGCTTATGGGAGGCGCACTTGACCAGTCTGCGGTACAT
      N  S  D  L  Y  C  D  I  M  G  I  M  L  P  R  V  V  P  F  E  E  K  P  S  L  W  E  A  H  L  T  S  L  R  Y  I

 109 ACATCATGGCTTGTGTCAGCAGgtaggattagctataagatttgaatctgcctaaccaattgtttcattcacagCGGTCAATTGAAGCCTGCCAAAAACTGTATAAT
      H  H  G  L  C  Q  Q                                                         R  S  I  E  A  C  Q  K  L  Y  N

 217 CTCATCCGAGCACAACCTTGCCGCCTGCAAGAGGAGTCGGACCACAAAATATACCTGGACATTCATTTGACCCACTTCAATGGCCTCCATGTGCTGCTGCAGAAGCAA
     L  I  R  A  Q  P  C  R  L  Q  E  E  S  D  H  K  I  Y  L  D  I  H  L  T  H  F  N  G  L  H  V  L  L  Q  K  Q

 325 AAACTACCTTTGGAAGCCACAAGTCACCTGTGCTATGCTCTGGAATCCCTGGGAGAACTGTTCGTGGCCATGAAGCAAAGGAAAATACATCAATGCGCACCGCTTCTA
     K  L  P  L  E  A  T  S  H  L  C  Y  A  L  E  S  L  G  E  L  F  V  A  M  K  Q  R  K  I  H  Q  C  A  P  L  L

 433 GTTCAACTAAACGAGAGTTTATTTGGCAAACGAAGTAGAACATTCTTTAAGTCCCTGAGCTTCTTGCCGTCGGAGAGTTTAGCCAAGATGTTCAACCCGCTGCTTATG
     V  Q  L  N  E  S  L  F  G  K  R  S  R  T  F  F  K  S  L  S  F  L  P  S  E  S  L  A  K  M  F  N  P  L  L  M

 541 CTCCTGGCCAGCAGCCCTACTTCAGACTTAGCCAACTTATTTCACGAGTACCTTAGTCTTACGTTTGCTCTCGTACAAATCGACATGTTTAGCCCGCAGTCCAATCAG
     L  L  A  S  S  P  T  S  D  L  A  N  L  F  H  E  Y  L  S  L  T  F  A  L  V  Q  I  D  M  F  S  P  Q  S  N  Q

 659 CAAATGTCATTGCAGCTGCTACGCGTGTGCAAGGAACTATTTCGCCAGGAATCAAACCTCAGCTATTCCCTACAGCTGATATACTACTATATCAAGTTAATCTATGTT
     Q  M  S  L  Q  L  L  R  V  C  K  E  L  F  R  Q  E  S  N  L  S  Y  S  L  Q  L  I  Y  Y  Y  I  K  L  I  Y  V

 757 CGGGAACCCACAGCCGACTTTAAACGTACCTACATTGACTTGTCGAGCAAATTTCAGCACTTCCTTGAGCACAAGGGAGCATCTCATGCCAAAGAACAGTGGCTTGCT
     R  E  P  T  A  D  F  K  R  T  Y  I  D  L  S  S  K  F  Q  H  F  L  E  H  K  G  A  S  H  A  K  E  Q  W  L  A

 865 GATTTTCTGGTGGCCATTCAATTGCTGCAGGTGCTTATCCATCAGAGTAGCAGTAAGTCGCAGAGCCCTTTTCAGTTATTTTGGCAGCAGTTTGACGGAGAGAGCAGT
     D  F  L  V  A  I  Q  L  L  Q  V  L  I  H  Q  S  S  S  K  S  Q  S  P  F  Q  L  F  W  Q  Q  F  D  G  E  S  S

 973 GTGGAGATCTATACAGCGCACTTTCAGTTGCTTCAGACGTGTGCTAGCTTGGCGGTTAATGTTACGAGGAGTCCCTTGGGCTGCAGTTGCACTCATGAGGCATGCAAG
     V  E  I  Y  T  A  H  F  Q  L  L  Q  T  C  A  S  L  A  V  N  V  T  R  S  P  L  G  C  S  C  T  H  E  A  C  K

1081 AGCGTGCGGAGGCACTGCATCTTGGCGTATGGATTGTGCGCATTGGATGCATATATTAATTGGAAATCGACTGAAGAGCAGAGAGCAAATGTGgtcggtatttaggaa
     S  V  R  R  H  C  I  L  A  Y  G  L  C  A  L  D  A  Y  I  N  W  K  S  T  E  E  Q  R  A  N  V

1189 tatatttaatctaattttgtgagaatttaataaacatttcaatgctattgcagAGTCCTCACAAACCCTTGCTGGGACTAGTCAAATACTCTATGGATGTGGCTAAGA
                                                           S  P  H  K  P  L  L  G  L  V  K  Y  S  M  D  V  A  K  T

1297 CCATGAAATGCTTGGGTCCCACCAGTGTGGAGCTCATCAAGCTAGTGCGCCAGCTGACATACGTGGCTGATCAGGTCACCTGTCCGGAGCAAATGTCCTTGTTGCCGC
       M  K  C  L  G  P  T  S  V  E  L  I  K  L  V  R  Q  L  T  Y  V  A  D  Q  V  T  C  P  E  Q  M  S  L  L  P  P

1405 CACTTTTGGAGCCACTGCAGAAGCTGCGACCTCTGATTGCCGACCAGGATATGAGTAGCTTACTCCGACGCCTCTTCAAAGCCAGCTACCATTGCAAGGATCCCAATA
       L  L  E  P  L  Q  K  L  R  P  L  I  A  D  Q  D  M  S  S  L  L  R  R  L  F  K  A  S  Y  H  C  K  D  P  N  M

1513 TGGCAAGTCGAATCCAAGCTAGCTATTTGGCCTCGATTACGAATCCCACCCGATTGAGATCTCAGATCTGTTTGTACTACCACAATTTGGGAAAAAAGGGCAATGAAA
       A  S  R  I  Q  A  S  Y  L  A  S  I  T  N  P  T  R  L  R  S  Q  I  C  L  Y  Y  H  N  L  G  K  K  G  N  E  I

1621 TCAGTAGGTGTGTCTACGAGTGGCATGAGTCCTCTCCACTACCTTTTCCTCTTACTCCGGACCAGACGAAACAGCTGTACGATATCGATTTCTTTGCCGCACTACACT
       S  R  C  V  Y  E  W  H  E  S  S  P  L  P  F  P  L  T  P  D  Q  T  K  Q  L  Y  D  I  D  F  F  A  A  L  H  Y

1729 ATTTGAGGAGTCCTTCTATGACTCATATGGAATCGCTAATTCGTTGCCGAATGAGTGACTACCATCTGGTACTTTTGGCCAGGCAAATGCGAAAGGATGCTTCAATTT
       L  R  S  P  S  M  T  H  M  E  S  L  I  R  C  R  M  S  D  Y  H  L  V  L  L  A  R  Q  M  R  K  D  A  S  I  S

1837 CGAAGAAGTGCTTGGAGGTTCATGCGAAGCTAAAGCAACAACGTACCCTCTGTCGAATGGAAGATTTGTGCCTGGGCCACGCAAGTGTTGGACTACTCCTGGATGCAC
       K  K  C  L  E  V  H  A  K  L  K  Q  Q  R  T  L  C  R  M  E  D  L  C  L  G  H  A  S  V  G  L  L  L  D  A  L

1945 TGGACGCTCAAAAAACCAAAGTTTCTACCAAGGAGATAACAGAAAACATGTTCGAGGAGCTGCTCCTCAGGCAGAATTTATGGCAGATGAATATACAAAGAGAGCAGC
       D  A  Q  K  T  K  V  S  T  K  E  I  T  E  N  M  F  E  E  L  L  L  R  Q  N  L  W  Q  M  N  I  Q  R  E  Q  R

2053 GTTTGGTCATCTATGCTAGCGAAGCTATCTCGGCTTTCAACAACTTCTTCAATCGAGCAGATCAAGAGCCATTGAGCAGCAATGAAACCTCTATTGATTGGGAGGCCT
       L  V  I  Y  A  S  E  A  I  S  A  F  N  N  F  F  N  R  A  D  Q  E  P  L  S  S  N  E  T  S  I  D  W  E  A  L

2161 TGATTGACGATGCCATCGCTGCTGCCAATGCACTTTCGAGTATGGGTTATCAATCCCAAGGGGATGATGCCTGGCTGCTGCTCCTGAGGATGGGTCGCTGGCTGGAAG
       I  D  D  A  I  A  A  A  N  A  L  S  S  M  G  Y  Q  S  Q  G  D  D  A  W  L  L  L  L  R  M  G  R  W  L  E  D

2269 ATCGTTTCACCTATCTCCGCGCCCTAAATCATTTTCTATCTCAGAATGAGGTCAGTACGCGATTGAGCCTGAATCTCTCCGAGGAAGTCGAAGTAGCAGAGGAATTGC
       R  F  T  Y  L  R  A  L  N  H  F  L  S  Q  N  E  V  S  T  R  L  S  L  N  L  S  E  E  V  E  V  A  E  E  L  L

2377 TGGATGATTTGTGGCCTCAACTGAAAAACGGAAAATTCTTTAAGCGACAGCAAACAACTGTAATGCTCTGTTTTTGTCACCTCGCCAGTTACTATGCCAGAATGGAAT
       D  D  L  W  P  Q  L  K  N  G  K  F  F  K  R  Q  Q  T  T  V  M  L  C  F  C  H  L  A  S  Y  Y  A  R  M  E  C

2485 GCCACAGCCATGCCCAGTTGCTTTTATTGCAAGTGGAACAACTTCGCGAAGAGTTTCCTGAAAGGCAAGGAAAAAGTGACATTGTATTGCTTACACTGCAAACAGTGC
       H  S  H  A  Q  L  L  L  L  Q  V  E  Q  L  R  E  E  F  P  E  R  Q  G  K  S  D  I  V  L  L  T  L  Q  T  V  R

2593 GCTTTCGACTAGGGTATCAGCAAAGGAAGACGACAAATTGCAAGCTGCCAACTCCCCTGCGACAATTGGACACTCTCTTGGACAATGTGCGAAGTTTCTGCAACCTAT
       F  R  L  G  Y  Q  Q  R  K  T  T  N  C  K  L  P  T  P  L  R  Q  L  D  T  L  L  D  N  V  R  S  F  C  N  L  S

2701 CCAGTTTAGATGGTGGCTCATTGCAGCTGCTTCTTTCGACTCTCGTCAGAGAAAGCACCGAGTCGTCTGCAAACAGATTAAGCGAACGACTGGCCTTCTCCAACATTG
       S  L  D  G  G  S  L  Q  L  L  L  S  T  L  V  R  E  S  T  E  S  S  A  N  R  L  S  E  R  L  A  F  S  N  I  A

2809 CGCTACATTTGGTCCTGCAATCTGGTTTGGCTTTGAGAACCATAGAGGTATTCCTCGCCTGGTTGTGGACCAATTTACAAATGGAAAATTTCGACAAGGCGCAATCGA
       L  H  L  V  L  Q  S  G  L  A  L  R  T  I  E  V  F  L  A  W  L  W  T  N  L  Q  M  E  N  F  D  K  A  Q  S  K

2917 AGTTGAGACTCGTCGAGCATTGTATGGGTATAAAACAGCTAAATCCAACGACTCGGCCAGAAAAGGAAGCGATTAAGGATCCAGCTATTAGTGATCTGGCAAGCAATA
       L  R  L  V  E  H  C  M  G  I  K  Q  L  N  P  T  T  R  P  E  K  E  A  I  K  D  P  A  I  S  D  L  A  S  N  M

3025 TGCATCTCCTCCAATTGGTGGAGCCAATCAGGAAACAGCATTTGTTAAATACGGCTTCGCCAAATCTACTCAGCATGCGACCACATAGCCCAAACCTCCAACTGGACT
       H  L  L  Q  L  V  E  P  I  R  K  Q  H  L  L  N  T  A  S  P  N  L  L  S  M  R  P  H  S  P  N  L  Q  L  D  L

3133 TGGATCGCTACCTGACACTGGATGAGGCGCCTTCGAATCTTCGAGAGAACTCTCAGCTGCAATGCTTGTACTTCGTAATGGGCTGTCTTCATGCACGTCTCCGCTTTC
       D  R  Y  L  T  L  D  E  A  P  S  N  L  R  E  N  S  Q  L  Q  C  L  Y  F  V  M  G  C  L  H  A  R  L  R  F  L
                                         EcoRI
3241 TTCAGCGGAAGAGTGAACAATTGGAAGAATTCTACGAAAAAGCGCATAGTTGGCTGCAGGAAAAACCAGTGGCGAGTAATGCTATGTGCTCCATGTTGCATGCCCAGC
       Q  R  K  S  E  Q  L  E  E  F  Y  E  K  A  H  S  W  L  Q  E  K  P  V  A  S  N  A  M  C  S  M  L  H  A  Q  Q

3349 AGCTCTTTCATCTGAACTATCTTCGCTTTATGCGAAAGCATGTAGAGGCTATATCAACGGCTCAATTGGGTCTCAAAATGCGGCCACGAGCGGTCGATATTAACTTTG
       L  F  H  L  N  Y  L  R  F  M  R  K  H  V  E  A  I  S  T  A  Q  L  G  L  K  M  R  P  R  A  V  D  I  N  F  E

3457 AGTTTAACTTCTTCGCTCAGCTAAAGACAGCTCAATCGGAGCTTAAACCCGTGGGCAAGGAAAAACCAAAGATCAAAACCCTTAGACGGGCTTTGGTATTCAATCTCT
       F  N  F  F  A  Q  L  K  T  A  Q  S  E  L  K  P  V  G  K  E  K  P  K  I  K  T  L  R  R  A  L  V  F  N  L  S

3565 CTCCGGAAGACAAGAAACGAACCGCAACCGGATCAGCTTCAGCAGTCAAGAATATGGCGGCCAAAGCCAAGCAGTCGGCCAAAAAGCCACCTCGGTTCAGAATTTACG
       P  E  D  K  K  R  T  A  T  G  S  A  S  A  V  K  N  M  A  A  K  A  K  Q  S  A  K  K  P  P  R  F  R  I  Y  E

3673 AGGAGTTGGAACTTCGACCACCGAGTGCTGTCAGTAACAGTAGCGGCGGCAGCGGAACAGAGAACACTCCACCTTCGGATCACGTGGATCTTAATGCCTGCCAAGCGA
       E  L  E  L  R  P  P  S  A  V  S  N  S  S  G  G  S  G  T  E  N  T  P  P  S  D  H  V  D  L  N  A  C  Q  A  I

3781 TCGAGATAAGCGACGACGACGATTTACCTTCAGTGGCCACGAAAAAGAACCAACCAAAAAGTAGAGATAAAGCTAAGCCCACGTCAAAAGCCTGTAAAGTCCTAACAT
       E  I  S  D  D  D  D  L  P  S  V  A  T  K  K  N  Q  P  K  S  R  D  K  A  K  P  T  S  K  A  C  K  V  L  T  L

3889 TGGACAACAGCTTGGAAATAGAAAAAACGCCAGCAAGAACTATGAGTGCACGGAGTACCAGAGCTAGGGCTCGCCAACCAGTAGAGACACCGAAGACAGCCACTCTTT
       D  N  S  L  E  I  E  K  T  P  A  R  T  M  S  A  R  S  T  R  A  R  A  R  Q  P  V  E  T  P  K  T  A  T  L  S

3997 CATCCAGGCGAACGAGGCGTCAGGTGTCGGAACCCCAGGCTCCTGAAACGGAGTCCACTAGCACACGCACACGTCACCGGCACTGAttagatagtagtctccttagtt
       S  R  R  T  R  R  Q  V  S  E  P  Q  A  P  E  T  E  S  T  S  T  R  T  R  H  R  H  *

4105 cttatttattcttattgaaaatgtcttggtatttaaatagatattgtatgtaagtgttactagaaattaaatatttccttttggtggcagggatagtagtgacgttat
                                               EcoRI
4213 acagatatcgttgctccaaaatccgcgctgaattc 4247
```

Figure 6.3 Comparison of noncoding nucleotide sequences from *D. melanogaster* and *D. erecta three rows* gene regions.

A. The first intron. Splice donator and acceptor sites are shown in bold.

B. The second intron.

C. 3' UTR. Shown in bold is the common termination codon and the *Eco*RI site in *D. erecta*.

A

```
D.m.      301 GTAGGAGTCGCTATAAGAATCTTCGAATCTTTCTAACCAATTCTCCCGTT
              |||||| | |||||||||   || ||||||   ||||||||| |  | ||
D.e.      131 GTAGGATTAGCTATAAGA..TTTTGAATCTGCCTAACCAATTGTTTCATT

          351 CACAG 355
              |||||
          179 CACAG 183
```

B

```
D.m.     1346 GTGGGTAT...........TTTTATCTAATTTTGTGGAATTTTAATGCTA
              || |||||           ||| |||||||||||||  | ||||||   |
D.e.     1174 GTCGGTATTTAGGAATATATTTAATCTAATTTTGTGAGAATTTAAT.AAA

         1385 CATTTCAATACTATTGCAG 1403
              ||||||||| |||||||||
         1223 CATTTCAATGCTATTGCAG 1241
```

C

```
D.m.     4251 TGATTAGATAGTAGTCTCCTTAGTTCGTATTTATTCTTATCGAAAATGTC
              |||||||||||||||||||||||||| ||||||||||||| |||||||||
D.e      4080 TGATTAGATAGTAGTCTCCTTAGTTCTTATTTATTCTTATTGAAAATGTC

         4301 TTGGTATTTAAATATATATATATATTGTGTGTAAGTGTTACAAAAGAGCT
              |||||||||||      ||| ||||||| |||||||||||| |
         4130 TTGGTATTTAA......ATAGATATTGTATGTAAGTGTTACTA.......

         4351 TTTGACATAAATTCATTGTTTTTTATCTTGTGGCAGGGACAGTAGTGACG
                       || | |    |||   | | |||||||||| ||||||||||
         4167 ........GAAATTAAATATTTCCTTTTGGTGGCAGGGATAGTAGTGACG

         4401 TTCTACAGATGTCCTTGCTCCAAAAACCGTGCTGAACCC 4439
              || ||||||| || ||||||||||| ||| ||||||  |
         4209 TTATACAGATATCGTTGCTCCAAAATCCGCGCTGAATTC 4247
                                               EcoRI
```

Figure 6.4 Comparison of the derived amino acid sequences of *three rows* from *D. melanogaster* and *D. erecta.*

Amino acid substitutions in *D. erecta* relative to *D. melanogaster* are shown beneath the full *D. melanogaster* sequence. Relative deletions in the *D. erecta* sequence are indicated with "-". Because of the limits of the *D. erecta* genomic clone no comparison is possible with the N-terminal 57 amino acids of *D. melanogaster* three rows.

```
D.m.    1 MSTDIATQLKGSRSDVEKVRKTVEAKFRELSGDGLPLRYEVNVLRHICLALKDNLHQNSD
D.e.      (                           ?                           )

       61 LYCDIMGIMLPRVVPSEEKPSLWEAHLSSLRYIHHGLFHQRSIEACQKLYNLIRQQPCRL
                         F           T         CQ               A

      121 QEESDYKIYLDIHLTHFNGFHVLLQKQKLPLEATSQLCYALESLGDLFAAMTQRQISLCA
               H             L               H         E  V  K  K HQ

      181 TLLVQLNESLFGKRSRSFFKSLSFLPSESLAKMFNALLMLLASSTSSNLANLFPECLSLT
          P               T                  P         PT D    H Y

      241 LALVQIDMFSPQSNQQMSLQLLRMSKELFRQESNLCYALQLMYYYIKLIFVREPTGDFKR
          F                       VC         S S   I       Y     A

      301 TYIDLSSKFQHFFEHKVASHAKEQWLADFLVAIQLLQVLIHQSNSKLQSPFQIFWQQFDG
                      L   G                          S  S     L

      361 ESSPEIYTAHFQLLQTCASLAVNITRSPLGCSCSHEACKSVRRHCILAYGLCALDAYINW
             V                   V         T

      421 KPAAEQRANVSPHKPLLGVVKYSMDVAKTMKCLGPTSVEIIKLVRQLTYVADQVTCPEQM
           STE              L                    L

      481 SVLLPLLEPLQKLRPLVADQDMSSLLRRLFKASSHCGDSNIACRIQASYLASITNPARLR
           L P            I                Y  K P M S             T

      541 SQVCLYYHNLGKKGTEIKRCVYEWHESTPLPFPLTPDQKKQLYDTDFFALLHYLRSPSTA
             I           N S         S          T     I             MT

      601 HMESLIRCRTSDYHLVLLARQMRKDDSISKKCIEVHDKLRQQRSLSRMDNLCLGHASVGL
                   M               A      L   A  K   T C  ED

      661 LLDALEAQKTKVSTKEITENMFEELLLSKNLWQMNIQREQRLVNYASEAISAFSNFFDRA
               D                     RQ              I         N   N

      721 DQEPLSANETSIDWEALIDDAIATANALSSMGYQSEEDDAWLLLLRMGRLLEDRFTYLRA
                S                A           QG            W

      781 LNHFLSQNEVSSRLNLKLGEEVEVAEELLDDLWPQLKNGKFFKRQQTTVMLCFCHLASYY
                     T  S N S

      841 ARMECYSHAQLLLLHVEQLREEFPERQGKSDIVLLTLQTVRFRIGYQQRKPTNCRLPTPL
               H        Q                            L      T   K

      901 RQLDILLDNVRSFCNLSSLDGGSLQLPLSTLVRESTESSANRLSERLSFSNIALHLVLQS
              T                     L                    A

      961 GLALRAIEVFLAWLWTNLQMESFDKAQSKLRLIEHCLGIKQLNPTSRPEKEAIKDVAISD
               T               N          V   M        T         P

     1021 LASNMHLLQLVEPIRKQQLLNMASPNLLKMRPHSPNPQLDLDRYITLDVAPANLRENSQL
                           H   T      S       L       L   E  S

     1081 QCLYFVTGCLHARLRFLQRNSEQLEEFYGRAHNWMQEKPPMSSALYPMLHAQQLYHLNYL
                M             K       EK   S L     A N MCS       F

     1141 RFARKHVEAISTAQLGLKMRSRAVDINFEYNFLAQLKTAQLELKPVGQDKPQVKILRRAL
            M                  P        F  F       S     KE  KI  T

     1201 VFNHSPEDKKRTATGSVSAVKNTASKVKQSAKKAPRFRIYEELELRPPSATSCSSSGGSG
             L            A     M A A      P                V -N

     1261 TENTPPSDHVDLNACQAIEISDDDDSPLVSTKKTQPKSREKAKPKATSKACKVLTLDNSL
                                   L S A   N     D   --

     1321 EIVETPTITTSTRSTRARLRQPVETPKTATLSSKRTRRQVLEAQAPETESISTRTRHRH* 1379
            EK  AR M A      A              R      S P       T
```

sequence motifs identified in *D. melanogaster*, the tyrosine kinase phosphorylation site at 119-126 (Table 3.1) is significantly altered in three rows of *D. erecta*. Just one of the four potential PEST sequences identified in *D. melanogaster* (at 1212-1233, Table 3.2) is disrupted by relative substitutions in *D. erecta*. One of the residues identical in the short region of similarity between three rows of *D. melanogaster* and p67^{nuc2} of *S. pombe*, the aspartic acid at 718 (Figure 3.6) is substituted with an asparagine in *D. erecta* (Figure 6.4).

Comparisons of the protein sequence databases with three rows of *D. erecta* once again reveal no regions of significant similarity to any known protein.

6.5 Discussion

The *three rows* gene of *D. melanogaster*, whose product performs an essential role in the evolutionarily conserved process of mitotic chromosome disjunction, may not be conserved even within the genus. Complementary sequences to *thr* cannot be detected in the genome of *D. virilis* by hybridisation under nonstringent conditions, both to genomic Southern blots, and genomic and cDNA libraries. *D. virilis* is estimated to have diverged from the *D. melanogaster* line about 60 mya at a major branch point that gave rise to the subgenera *Drosophila* and *Sophophora* (Beverley and Wilson, 1984; Spicer, 1988) (Figure 6.1).

The homologue of *thr* has been cloned from a Sophophoran species, *D. erecta,* proposed to have shared a common ancestor with *D. melanogaster* 17 mya (Lachaise et al., 1988). The nucleotide and derived amino acid sequence of *D. erecta thr* are both 88.3% identical to *D. melanogaster*. Relative to the other *D. erecta* homologues of *D. melanogaster* genes reported (Table 6.1) *thr* appears to have been poorly conserved in the course of evolution. Of the three genes, *transformer*, *Sgs-3* and *Ref(2)P* (Table 6.1), whose products exhibit more divergence, all are distinguished by regions of repetitive, low complexity, amino acid sequence, seemingly capable of tolerating polymorphism. Relative to the proteins without sequence degeneracy, alcohol dehydrogenase and α-amylase, three rows is clearly the least constrained (Table 6.1).

Even within *D. melanogaster*, *thr* sequence is demonstrably prone to variation. Nucleotide polymorphisms, some of them leading to amino acid substitutions, have been detected in short regions of common sequence derived from the Canton-S and *iso-2* strains (Section 3.4). Still further polymorphisms have been revealed by restriction analysis of cDNA and genomic clones (J. Camerotto, pers. comm.) and in the other report of *thr* sequence (Philp et al., 1993).

The homologue of the *tra* gene has been isolated from *D. virilis* by low stringency hybridisation (O'Neil and Belote, 1992). By the criterion of the relative sequence divergence of their homologues in *D. erecta* (Table 6.1), the *tra* gene is less evolutionarily conserved than *thr*. So why is it not possible to detect the *thr* homologue in *D. virilis* in the same manner as *tra*? One possible explanation pertains to the amino acid content of three rows and the consequent degeneracy of the codon dictionary. 30.8% of the amino acids in three rows can be encoded by 6 different codons (R, L and S), compared to 19.3% in the average content of proteins (Dayhoff, 1978) (Table 3.1). This gives considerable potential for nucleotide sequence divergence, by genetic drift, without resultant amino acid variation.

Alternatively, the failure to isolate a homologue in *D. virilis* by hybridisation may reflect divergence of the protein sequence. Evidence that the protein is not highly conserved comes from the failure, in preliminary experiments, to immunodetect the homologue of three rows in whole mounts of *D.virilis* (data not shown). The ability to detect homologous sequences in *D. virilis* could be further hampered if the rate of sequence divergence in three rows has varied during the evolution of the genus.

Despite the evidence from hybridisation approaches it remains likely that *thr* homologues will be found in *D. virilis*, and further afield, and that their characterisation will be informative. Strategies for the isolation of more diverged homologues will be discussed in the next chapter.

Chapter 7 (Telophase): Summary and prospects for future work

7.1 Summary

three rows, a gene previously only defined by its distinctive mutant phenotype, has been isolated and its coding sequence and product characterised. The encoded product is unlike any protein known to date and therefore defines a previously unknown activity essential for mitotic chromosome disjunction.

The temporal and spatial pattern of *thr* expression has been elucidated. The expression pattern provides an explanation for why the mutant phenotype becomes apparent in cycle 15, a complete cycle later than the initiation of zygotic transcription of *thr* in wild-type embryos. Maternally derived mRNA appears to be actively degraded at the time of cellularisation, and three rows function in cycle 14 in homozygous mutants is believed to be conferred by persistant protein derived from the maternal complement.

Immunostaining of embryos with three rows specific antibodies has revealed a dynamic, cell cycle dependent pattern of localisation, consistent with the defect in chromosome disjunction observed in mutants. Three rows, undetectable in metaphase, is localised to the chromosomes in anaphase, initially to the region of the presumptive kinetochore, where it may function in chromatid separation or movement.

The isolation and characterisation of homologous sequences from *D. erecta* indicates that, despite being essential in *D. melanogaster* for the universal process of chromosome disjunction, *thr* is not highly conserved in the course of evolution.

7.2 Future work

7.2.1 Further characterisation of mutant phenotype

Although *thr* mutants are defective in chromosome disjunction in cycle 15, subsequent nuclear events such as chromosome decondensation and DNA replication in

cycle 16 have been observed to proceed (D'Andrea et al., 1993). It remains to be determined, however, whether these downstream events occur on a normal schedule. In the phenotypically identical *pimples* mutant, an elevated frequency of mitotic Figures has been observed (Smith et al., 1993), consistent with a prolongation of mitotic organisation. Similarly, homozygous *thr* mutant embryos have a high mitotic index, particularly in cycle 16 (data not shown), suggesting a delay in downstream events.

How might mutations in *thr* hinder cell cycle progression? It has been established that degradation of cyclins A and B occurs normally in cycle 15 in *thr* mutants (D'Andrea et al., 1993). Because of the causal relationship between cyclin degradation and MPF inactivation (Murray et al., 1989) it is assumed that failure of MPF inactivation is not responsible for the inferred cell cycle delay. However the consequences of the *thr* mutant defect on other regulatory events is unknown. In particular, the activity of specific phosphatases and kinases has been implicated in regulating late events of mitosis. Requirements for both PP1 (Axton et al., 1990) and PP2A (Mayer-Jaekel et al., 1993) activity in chromosome segregation in *D. melanogaster* have been revealed by the phenotype of mutants. The product of *polo*, a member of a conserved family of protein kinases (Clay et al., 1993), is necessary for late mitotic events (Llamazares et al., 1991) and its activity appears to peak in anaphase/telophase (Fenton and Glover, 1993). Determination of the patterns of accumulation or activity of protein phosphatases, or of polo kinase, in *thr* mutant backgrounds, may reveal the regulatory events which result in the apparent cell cycle delays post cycle 15.

Chromosome disjunction can be conceived of as two discrete events: the separation of sister chromatids and, subsequently, their polewards movement. It remains to be determined which of these two aspects of chromosome disjunction are defective in *thr* mutants. Some spindles have been observed in *thr* mutants in which chromosome separation has commenced but is apparently never completed (D'Andrea et al., 1993). These observations imply that it is sustained polewards movement that is defective in mutants, not sister chromatid separation (SCS).

Chromosome movement in anaphase is clearly a MT dependent process, whilst SCS may simply result from the dissolution of bonds between chromatids. If so it should

be possible to establish specifically whether SCS proceeds, in *thr* mutants, by treatment with MT depolymerising drugs, such as colchicine, followed by hypotonic shock. Unfortunately SCS, which has been observed in other systems following treatment with MT depolymerising drugs (Rieder and Palazzo, 1992), has been reported not to occur in *D. melanogaster* (Gonzalez et al., 1991). This may be a product of the demonstrated dependency, in *D. melanogaster*, of cyclin B degradation on an intact spindle (Whitfield et al., 1990). Accordingly, dissolution of the spindle in *thr* mutants will inhibit cyclin B degradation and thus block anaphase initiation. Without entry into anaphase it may not be possible to test explicitly for SCS by spindle disruption.

Despite the failure to progress into anaphase it may still be informative to treat *thr* mutant cells with colchicine, to test for the phenomenon of precocious sister chromatid separation (PSCS) (Smith et al., 1985). In *l(1)zw10* mutant neuroblasts following treatment with both MT depolymerising, and stabilising, drugs 30-60% of cells exhibit PSCS compared to 2-7% in treated wild-type brains.(Williams et al., 1992; Williams and Goldberg, 1994). Cyclin B levels remain high in both wild-type and mutant cells treated with colchicine suggesting PSCS is not a consequence of defects in a *mad/bub* type of checkpoint mechanism (Section 1.1.7). Rather it has been suggested that defects in *zw10* abolish the dependency of sister chromatid separation on spindle integrity by a novel mechanism that is independent of MPF activity (Williams and Goldberg, 1994).

Another aspect of *thr* that remains to be investigated phenotypically is its implied role in nonembryonic proliferation. *thr* transcripts have been detected in all postembryonic life cycle stages marked by significant levels of mitotic proliferation (Section 4.4). The requirement for *thr* in imaginal proliferation can be tested by mosaic analysis, using the FLP/FRT system (Golic and Lindquist, 1989) to generate adults with clones of cells in which the *thr* gene is deleted. The generation of individuals with sections of the adult cuticular structures missing or hypotrophied would be evidence of an essential role for *thr* in postembryonic proliferation.

7.2.2 Further analysis of expression

Polymorphisms in the length of both the 3' and 5' UTRs have been detected in the analysis of transcription products. It may be informative to determine how these variants arise and what specific function, if any, they perform.

The polymorphisms in 3' UTR length revealed in sequence analysis of cDNA clones appear to arise from alternate polyadenylation signals. The isolation of both forms from a 4-8 h embryonic cDNA library may indicate correspondence to maternal and zygotic forms of transcript. This could be verified by utilising of 3' sequences specific to the longer cDNA form as a probe to a population of fixed embryos that includes syncytial and cellularised individuals. The maternal form would be predicted to be abundant in syncytial embryos, but undetectable in cellularised embryos in all but the pole cells, whilst the pattern of detection of the zygotic form would be essentially complementary.

The length polymorphisms detected in the transcript 5' UTR by primer extension analysis also merit further investigation. cDNA clones of these variant forms could be derived by RACE PCR and the mechanism of their generation, by processing or by initiation of transcription at alternate sites, determined by sequence comparison with 5' genomic regions. Evidence for developmental stage specificity has come from primer extension analysis, with the detection of a 58±5 nt UTR transcript only in early embryos (Section 4.4). Developmental stage specificity of these 5' UTR variants could be resolved, as for the 3' forms, by the application of specific probes for *in situ* hybridisation.

In situ hybridisation to *thr* mRNA in early embryo whole mounts has revealed distinctive spatial and temporal patterns of localisation (Section 4.5), the mechanisms of which remain to be determined. Three noteworthy, possibly inter-related, patterns of *thr* mRNA detection were discerned: perinuclear localisation in syncytial embryos, the inferred specific degradation of maternally derived message at the time of cellularisation, and the persistence in pole cells.

The perinuclear distribution and pole cell accumulations are similar to those observed for transcripts of cyclin B (Whitfield et al., 1989; Lehner and O'Farrell, 1990b; Raff et al., 1990). The perinuclear distribution of cyclin B mRNA is known to involve MTs (Raff et al., 1990) and recognition sequences in the 3' UTR (Dalby and Glover, 1992)

although the precise mechanism is unknown. The same, or possibly different, 3' UTR sequence elements are responsible for the posterior pole accumulation of cyclin B mRNA and its persistence in pole cells late in development (Dalby and Glover, 1993). Comparison of the 3' UTR sequences in *thr* and cyclin B mRNAs failed to detect sequence similarities (data not shown). However, if cyclin B and *thr* message do share a common mechanisms of localisation these could conceivably be conferred by shared secondary structures, not necessarily matching nucleotide sequences.

The precipitous decline in the level of maternal message at the time of cellularisation is a characteristic *thr* shares with a number of other genes including *dorsal* (Steward et al., 1988), *stg* (Edgar and O'Farrell, 1989), *twine* (Alphey et al., 1992) and *cycE* (Richardson et al., 1993). The mechanism of this apparently active process of degradation is unknown and is worthy of further investigation.

7.2.3 Immunodetection

The immunostaining of whole mount embryos has revealed a distinctive pattern of localisation that is consistent with the mutant phenotype and offers some functional insights. The antibody can be further exploited to elucidate the function of *thr* in a number of ways.

The question of three rows role in meiotic, as well as mitotic, proliferation can be resolved by immunostaining of adult ovaries and testes. A similar pattern of localisation in meiotic divisions in oocytes and spermatogenic cysts would suggest a function in all forms of chromosome disjunction. In particular given the apparent similarities to chromosome disjunction in mitosis, the participation of three rows in meiosis II would not be unexpected. Immunostaining of ovaries may also reveal if three rows is part of the maternal complement in nurse cells, and if it functions in gametogenesis associated mitotic proliferation, for example, in follicle cells.

Conversely, one might expect the absence of three rows in tissues undergoing polyteny. Institution of the endo cell cycle involves modifications in the patterns of expression of certain genes, particularly the switching off of genes that catalyse entry into

M phase (Smith and Orr-Weaver, 1991). Three rows, with its function apparently restricted to mitosis, would be one of the predicted targets of this down regulation.

Although clearly essential for chromosome disjunction, the detection of three rows throughout mitosis could imply a role in earlier stages of mitosis. One method of defining the temporal requirement for three rows is to observe the effect of injecting specific antibodies into syncytial embryos, or tissue culture cells. A requirement for function strictly in anaphase would be predicted to phenocopy the mutant, whilst an earlier requirement may perturb or block events in prophase or prometaphase.

Immunostaining has revealed the rapid mobilisation of three rows to the presumptive kinetochore region of the chromosomes, at the commencement of disjunction. In order to confirm that three rows does localise to the kinetochore, double immunostaining experiments could be performed with antibodies against established kinetochore proteins, such as zw10 (Williams et al., 1992) or S5-39 (Kellogg et al., 1989).

The abrupt appearance of three rows on the chromosomes at the commencement of disjunction is correlated temporally with the inactivation of MPF at the metaphase-anaphase transition. To test if three rows localisation is *dependent* on MPF inactivation, wild-type embryos or larval brains could be immunostained following treatment with colchicine. Colchicine treated *D. melanogaster* cells arrest prior to anaphase with high levels of MPF, due to the dependency of cyclin B degradation on MT integrity (Whitfield et al., 1990) (Section 7.2.1). If dependent on MPF inactivation, three rows localisation to chromosomes should not be observed in colchicine treated cells. Similar experiments have demonstrated that zw10 translocation is not dependent on MPF inactivation, as it continues to accumulate at the kinetochore in mitotically arrested cells (Williams and Goldberg, 1994).

If, as the present study has indicated, three rows is not greatly immunogenic it may be possible to exploit the antigenicity of other protein epitopes to enhance immunodetection. "Epitope tags" consist of short polypeptides, to which highly specific (usually monoclonal) antibodies are directed (Geli et al., 1988). Chimaeric proteins comprising the epitope tag fused at either the N-terminal or C-terminal end to the protein of interest, can be engineered by recombinant techniques. In *D. melanogaster in vivo*

expression can be induced following genomic insertional transformation and the pattern of localisation of the chimaeric protein detected with the epitope tag directed antibody. To confirm that the function and localisation of the chimaeric protein reflects that of the native form, the ability to complement the mutant phenotype would first need to be demonstrated.

7.2.5 Isolation of a diverged homologue

The isolation of a *thr* homologue from *D. erecta* whose product exhibits 88.3% identity to *D. melanogaster* three rows is indicative of a rapidly evolving product. Such a level of divergence is not sufficient to uncover conserved, functional domains, although it does suggest that the isolation and characterisation of a *thr* homologue from a more distantly related member of the genus will be informative. Unfortunately *thr* is not detectable by hybridisation under nonstringent conditions in the species of choice for this form of analysis, *D. virilis*, estimated to have diverged from the *D. melanogaster* line 60 mya. Accordingly other methods will have to be employed to isolate a more diverged *thr* homologue.

One strategy is to exploit the evolutionary conservation of a nearby gene. If a gene in the vicinity of *thr* can be identified, that is conserved in *D. virilis*, it can be used as a probe to initiate a chromosomal walk in *D. virilis* to eventually isolate homologous sequences to *thr*. Such a strategy assumes that the genomic structure in the vicinity of *thr* has been conserved during the estimated 60 my of evolution. For this reason it is preferable that the starting point of the walk be close, to minimise the chances of chromosomal rearrangements having occurred in the intervening genomic region during the period since divergence. On the basis of hybridisation to the "genus blot", sequences complementary to the cDNAs, UJB6 and UJ4c, representing the transcription units immediately adjoining *thr* (Figure 3.1), appear not to be any more conserved than *thr* (data not shown). Fortunately, the more distal gene *grh*, the starting point for the chromosomal walk that resulted in the isolation of *thr* (Figure 3.1), has been conserved during the course of *Drosophila* evolution and the homologous sequences isolated from *D. virilis* (S. Bray, pers. comm.). These sequences will be used in future work to span the region in *D. virilis* that corresponds to the approximately 18 kb *grh-thr* interval in *D. melanogaster*.

An alternative strategy for isolation of a *thr* homologue in *D. virilis* would be by PCR amplification with degenerate primers. A number of primers could be designed that correspond to regions of amino acid sequence, conserved between *D. melanogaster* and *D. erecta*, with low levels of degeneracy in coding sequence. These could be used in varying combinations in an attempt to derive a PCR product from genomic or library DNA sequences of *D. virilis*. The PCR products would be sequenced and the efficacy of this approach confirmed by detection of derived amino acid sequence homologous to three rows of *D. melanogaster*. If this strategy does not yield an homologous sequence from *D. virilis* a more incremental approach through the genus may have to be adopted, starting with a less diverged species such as *D. ananassae* (Figure 6.1).

7.2.6 Identification of interacting proteins

Most proteins in the cell do not function in isolation but as integral parts of multisubunit complexes, or as components of signalling and dependency pathways. Determination of the identity of proteins that interact with a newly characterised gene product may reveal the biochemical basis for its function. This is particularly so in the case of a "pioneer" protein like three rows for which immunolocalisation has not given a clear indication of its role. A number of methods exist for identifying interacting proteins. Each technique has its strengths, as well as potential to identify spurious interactions. A systematic analysis should exploit a number of independent strategies to unambiguously indentify interacting proteins. Most of these methods can be utilised in screens to identify previously unknown interactors, or in a candidate approach to test for interaction with proteins with similar patterns of immunolocalisation or those encoded by genes with phenotypic similarities. In the candidate approach genes that would be worthy of investigation would include *pimples*, mutants of which have a phenotype indistinguishable from that of *thr* (Smith et al., 1993), as well as *l(1)zw10*, *qrt*, *aar*, *rough deal* and *lodestar*.

7.2.6.1 Genetic screens

The application of mutational screens, is a well established and reasonably reliable means of identifying interacting gene products. Mutants in secondary loci, can be isolated

and characterised that are capable of either enhancing or suppressing the severity of a mutant phenotype. Because of the timing and lethality of its phenotype *thr* is not an ideal mutant for this type of screen. In particular, identification of enhancers of phenotype, and recovery of the strain is effectively impossible. Potentially more fruitful is a screen for second site suppressors that takes advantage of the partial function in the thr^{IIV} allele. The success of such an approach is still problematic, because an F_1 screen demands that a heterozygous second site suppressor can compensate sufficiently for loss of *thr* function, to ensure survival to adulthood. Because of the potential for the detection of nonspecific effects, further characterisation of the secondary locus would be required to establish the veracity of the interaction.

7.2.6.2 Immunological approaches

Specific antibodies can be exploited in a number of ways to ellucidate protein interactions.

Antibodies can be used to examine the interaction of three rows with candidate interactors, in particular the *pim* product. Application of the anti-three rows antibody to *pim* homozygotes will reveal if three rows localisation, particularly at anaphase, is dependent on *pim* function. Mutants of other candidate genes could also be examined. Such an approach has demonstrated that mutation of *aar* or *rough deal*, but not *lodestar*, can affect the localisation of zw10 (Williams and Goldberg, 1994).

Using a reciprocal approach antibodies against proteins that are candidate interactors with three rows could be used as probes to *thr* mutant embryos. In particular the monoclonal antibody Aj1 (Frasch et al., 1986) has a similar pattern of immunolocalisation to that of three rows. In the absence of a corresponding gene or mutant for Aj1, this is presently the only method of establishing an interaction.

Antibodies can also be employed to demonstrate interaction between proteins in cellular extracts by immunoprecipitation and immunoaffinity chromatography. Proteins that form stable complexes *in vivo* can be coimmunoprecipitated with antibodies against one of the components of the complex. Interacting proteins can then be visualised by electrophoresis, and their identity determined by purification and protein microsequencing,

or by western analysis with antibodies against candidate interacting proteins. Candidates for which antibodies exist, are the aforementioned Aj1 antigen and zw10. Genuine interactions can be confirmed with the reciprocal approach; detecting three rows in complexes immunoprecipitated with antibodies against other proteins in the complex.

Proteins that associate with three rows in multicomponent complexes can also be purified by immunoaffinity chromatography. Low affinity antibodies against the centrosomal protein DMAP 190 have successfully been used to identify 10 major interacting proteins in cellular extracts, including one protein shown by immunostaining to be centrosomally localised (Kellogg and Alberts, 1992)

7.2.6.3 Exogenous reconstitution of interactions

Other methods of identifying interacting proteins rely on the detection of interactions between components that are exogenously expressed and reconstituted.

One such technique is the screening of an expression library using, as a probe, the protein of interest (Defeo-Jones et al., 1991). The protein will bind to membrane immobilised protein, produced by individual clones of an expression library for which it has an affinity, and can be detected immunologically or radioactively. Although the interaction occurs entirely *in vitro*, and may have considerable potential to reveal artefactual associations, this method has been used successfully to isolate proteins that interact with retinoblastoma (Defeo-Jones et al., 1991).

Another method for identifying interacting proteins, that is finding much favour, is the "two hybrid" system (Fields and Song, 1989). Essentially, this system relies on the reconstitution of a transcriptional activator to drive the expression of a selectable marker in yeast cells, cotransformed with expression constructs of the gene of interest, and an appropriate cDNA library. The two hybrid system be used to "fish" for previously unknown interactors in a cDNA library screen, or to test for interaction with the products of candidate genes. Furthermore, once an interaction has been detected, the system can be employed to define precisely the regions of the proteins required for the interaction.

7.3 Conclusion: *three rows* and its likely contribution to current issues in mitosis

Despite the apparently rigorous model for the operation of the mitotic oscillator and the detailed cytological descriptions of events in mitosis we are still remarkably ignorant about the molecular mechanisms involved in the formation of a bipolar spindle, the structural transformations that chromosomes undergo during mitosis, and their movements. In particular, major foci of attention in contemporary research on mitosis are the questions of the relative significance of molecular motors and microtubule dynamics to force production, the signalling event(s) that trigger chromosome disjunction in anaphase, and the mechanism of the dissolution of the bonds between sister chromatids at the commencement of disjunction.

Because of its essential role in chromosome disjunction; its product, unlike any other known to date; and its distinctive pattern of subcellular localisation, *three rows* seems well placed to contribute to the resolution to these dilemmas. Elucidation of the biochemical function of *three rows*, by application of some or all of the experimental strategies outlined in Section 7.2, will identify a previously unsuspected activity required for chromosome disjunction and contribute to an understanding of the mechanism of mitosis in all eukaryotes.

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